

Making budgets in a straitjacket

The new US president has signalled how he would have spent money if there were more of it, and has singled out public education. The academic community should see that the message is amplified.

PRESIDENT George Bush's heart is in the right place, but his pockets are filled with lead. That is the first conclusion about the new US presidency to be drawn from the extraordinary occasion last week when the new incumbent, with beginner's bustle, rolled together the traditional State of the Union Address with the much-awaited explanation of how his budget will differ from that submitted by his predecessor to the US Congress just a few weeks ago (see *Nature* 337, 104 & 200; 1989). Bush said all, or nearly all, the right things, but does not command the resources to make his wishes come true.

The new administration's difficulties have been plain for several months. To his credit, the new president did not hide from them last week. The formal constraint is so to manage planning for the next financial year (beginning on 1 October) that the statutory calculation in August of next year's deficit will come out at less than the \$100,000 million allowed by the Gramm-Rudman rules.

The less calculable but more serious objective is to manage affairs so as not to disturb those elsewhere on whose willingness to continue lending to the United States depends the remarkable stability and growth of its economy. The new men at the White House have calculated that continued economic growth (an optimistic 4.5 per cent next year) will give them, in a year in which tax revenues increase above \$1 million million for the first time, nearly \$88,000 million extra to play with. No less than \$64,000 million of that will be thrown into the expected spending gap, leaving a relatively small amount to meet extra inescapable costs. No wonder that the new president last week could make only token financial gestures towards the goals he has set himself for the four years ahead.

That does not mean that the goals are negligible. Under the rubric of US competitiveness, the Bush administration avows its support for science and technology (extending the tax credit on research and development spending by companies, affirming that the budget of the National Science Foundation must be twice that in 1988 by 1993 and even blessing the Superconducting Super Collider by name). Under the rubric of the "kinder and gentler society" which mocks the recollection of last year's bruising election campaign is a variety of environmental programmes that promise well (but will cost money only in future).

New programmes

The war on drugs is to be prosecuted with vigour, but again with real spending deferred. And the budget for next year includes a clutch of entirely novel spending programmes in support of public education in the United States which are to be judged by what they say about the president's intentions, not for what they will or even might accomplish.

While the US Congress may (and probably will) think differently, it will seem to many to be cruel that this brave talk should be heard when the US government is more constrained than ever by the lack of funds. Yet the truth is even more cruel than that. For the point seems to have been reached, in the brave history of the United States, when the future is constrained not simply by the gap between the US Treasury's spending and its disposable income, but by the gap between the ambitions of

the United States as a whole and its disposable intellectual resources. For the state of public education in the United States, acknowledged for at least the past decade to be dreadful, should now be recognized as the most serious obstacle to the attainment of this and future new presidents' forgivably optimistic goals.

The circumstances are amply catalogued. The study of public education commissioned at the end of the Carter administration vividly drew attention to some of the more obvious problems — the difficulty of staffing engineering schools except by relying on graduate students from overseas, for example. By 1983, the Reagan administration's own contribution to the catalogue of neglect (called *A nation at risk*) declared that "a rising tide of mediocrity . . . threatens our very future". For a little while thereafter, several progressive states (which shoulder direct responsibility for education) made headway with schemes for improving the condition of elementary and secondary schooling, many of which persist. Yet the signs are that public education in the United States continues to deteriorate. Two years ago, a committee of the Carnegie Corporation under Dr Lewis Branscombe (then at IBM, now at Harvard) made the same point about the dependence of economic prosperity on the quality of young people's education, and chilled its audience with unflattering comparisons between the performance of children from the United States and elsewhere (in *A nation prepared*).

Promise of success

Since then, other more sombre truths have come to light — for example, the failure of 76 per cent of black students to graduate within six years from the universities they had begun to attend in 1980. It is no wonder that President Bush, like most candidates in last year's several election campaigns, is vowing again to fulfil the traditional promise of the United States that all newcomers, native-born or otherwise, will have an equal crack at success.

How well has he begun? The spattering of new programmes offered last week will not by themselves make much difference either way. Awarding prizes to successful schools and teachers may marginally encourage the others, while the plan to let each member of the Congress nominate a recipient of a \$10,000 scholarship in science or mathematics will at least set senators and congressmen wondering about the causes that have thrust this patronage upon them. What matters more is that the new administration has declared its readiness to help stop the rot. Will the intellectual community in the United States be willing to seize the opportunity thus presented?

On the face of things, there is little that can be done. Public education, after all, is the responsibility of state governments, while one of the more obvious impediments to reform — the desperate shortage of able teachers — seems intractable unless all teachers are paid more, which would cost fortunes that no longer are easily manufactured. In any case, in the United States as elsewhere, academics working outside the elementary and secondary sectors of education have tasks of their own with which to grapple. The competition for research grants is as fierce as ever, but the administrators are now even more determined that overhead costs should be recovered. Yet like the US economy, US higher education rests on the now-shaky founda-



tion of elementary and secondary education. The all-too-realistic fear in the United States that the economic competition with Japan (and even Europe) will eventually be lost stems from the acknowledged poverty of public education. Should not universities and other institutions of higher education recognize that their survival will even more literally turn on the future health of elementary and secondary education?

But what can research and higher education accomplish in such foreign fields? If it were that the problems of public education in the United States could be solved by spending money, it would probably be a better investment in the future to scrap the Superconducting Super Collider, or the extra shuttle flights, or even the National Science Foundation's doubled budget, for the sake of the improvement that those funds might buy. But money by itself is not sufficient. The more urgent needs are for a better understanding of how schools function well, for a school curriculum which is at once stimulating and improving, for a general appreciation of the particular importance of the most elementary education and for plain speaking about the seriousness of the difficulties created by the continuing impoverishment of the school system. Who but those in the academic community can do all these things? And how can they be neglected when it is as plain as at present in the United States that sufficient attention paid to public education would solve many of the other problems preoccupying the new president? □

Fraud on the agenda

The US Institute of Medicine recommends how malpractice in research should be controlled.

LAST week's report (see page 588) from the Institute of Medicine in the United States is one of the first by an academy or society representing a substantial part of the research community anywhere to face up to the problems of misconduct in the practice of research. That is not nearly as reprehensible as it may seem. Although the past decade has been peppered with lurid accounts of how apparently diligent researchers have pulled wool over the eyes of colleagues, either by the downright fabrication of data or by plagiarism, it is natural that leaders of the research profession should have been slow to respond.

Something akin to charity first prompts the belief that isolated scandals have been made conspicuous by contrast with the general practice, which is honourable. Then follows the proper fear that formal inquiry will engender the general opinion that research is shot through with malpractice. In the circumstances, the Institute of Medicine has been courageous in breaking new ground with its investigation. It is forgivable that its conclusions should be as tentative as they are.

The underlying difficulty is, of course, familiar — that there is no way of telling how common malpractice may be. Banks may hope to fix upper limits on the extent of embezzlement by careful accounting practice, for their losses on this account must always be less than the funds they cannot account for, but there is no arithmetical yardstick by which the total volume of scientific discovery is conserved. So may not the publicized cases of outright misconduct be simply the tip of a much larger iceberg? While there is no means by which the magnitude of the problem can be assessed, that suspicion cannot be flatly denied. What that, in turn, implies is that the damage done to the reputation of the research profession is certain to be much greater than is just. Researchers should not be too indignant on that score; even the most honest stockbrokers have been given a rough time because of the cases of insider trading brought to light in the past few years. The research profession naturally has an interest in regaining its reputation. A little reflection should show that its paymasters, usually governments, should be similarly inclined. How is that to be accomplished? Last week's report points to several ways which are none the worse for being obvious.

Universities should be more diligent and vigorous in their

investigation of complaints or even suspicions, as should be grant-making institutions, while journals and professional societies also have their parts to play. Inevitably, the report muses on the incentives for misconduct and, concluding that competition in research and the pressure to publish are in part responsible, asks that something be done to blunt the edges of these pressures. Nothing much is new; the novelty is that it has been said by a respected but independent research academy. But the outcome is not a recipe for what should now be done.

Yet everybody working in research must know the answer. First, research is a profession whose integrity is, by definition, of astounding integrity; if it were otherwise, so that all published data and calculations were suspect, the notion that people can see further than others, in Newton's simile, by standing on the shoulders of others (giants or otherwise) would be falsified, and the scientific enterprise would long since have ground to a halt.

The torrent of discovery in the past few decades is in itself a proof of the integrity of the fabric of science. That is what nervous politicians must be made to understand. But, second, precisely because of the general appreciation of the integrity of the research profession, it is natural that people whose inclinations are less honourable should be attracted to it. In research, the problem is further magnified by unavoidable competition and by the circumstance that people are not required to behave well towards each other. Then, third, because discovery is the process of coming to grips with what people do not understand, mistakes are unavoidable and frequent — and cannot always be unambiguously distinguished from malpractice.

These sociological observations, however trite, have a bearing on the problem the Institute of Medicine (for one) would like to solve — that of minimizing the incidence of malpractice. The first conclusion must be that the incidence can never be absolutely zero, and that attempts to make it so can only bring science to a halt. Second, the people best placed to tell when observations have been improperly carried out or described are the close colleagues and competitors of the author or authors. That no doubt explains why committees of inquiry in this field have had such a poor track record in the past few years.

University departments or their equivalents in other kinds of organizations are better placed to sense when things have gone awry, and to take remedial action quickly. That they have been less effective than might have been expected in the past few years reflects two uncomfortable tendencies — the growing importance of the principal investigator as an autonomous unit in the conduct of research, and the dominance of his relationship with an external source of funds over that with more immediate and discerning colleagues. While principal investigators as such have not been conspicuous among malpractitioners — more often, it has been the hands they hired — putting back that clock completely would be impracticable, and probably unwise. But there is a case for arranging that principal investigators should more often than at present be less dependent on an external grant-making agency, and more dependent on their colleagues locally. At worst, good researchers who are not good managers might be helped to deal perceptively with their assistants.

The Institute of Medicine's report follows most other lines of argument than this. There should be more guidelines, university officials with special responsibility for "the responsible conduct of research", a special office at the National Institutes of Health (and presumably other agencies) to investigate complaints and even courses of instruction for beginners in research. In reality, there is at least as strong a case for believing that grant-making agencies should be less, not more, immediately involved: they should rather confine themselves to making sure that grant-recipients' institutions arrange that their funds are spent responsibly. And most grant-making institutions in the United States are too close to Congress, and too remote from where the action is, to function effectively and quickly in the prevention of misconduct. The best they can hope to do is to function judicially, after the event, which is a heavy-handed business. □

British research learns how to spend extra

- New interdisciplinary centres under way
- Three-year increases in science budget

London

ONE of the most recent innovations in the British pattern of research support, the interdisciplinary research centre (IRC) is to be the subject of a major review after the British government has expressed caution about the programme. The review, which is to begin this month and be complete by the summer, will cover the selection of topics on which the centres work, their management, links with industry and their evaluation.

This development was announced with the publication last week of the annual advice from the Advisory Board for the Research Councils (ABRC) to the Department of Education and Science about the allocation of the science budget for the years ahead. The occasion is especially important this year because £340 million will be added to the science budget over the next three years.

For next year, the extra money amounts to £107 million, bringing the total for 1989–90 to £825 million. Of the extra, a quarter will be absorbed by extra allocations already made to the British Geological Survey, the British Antarctic Survey, CERN (the European High-Energy Physics Laboratory), directed research on AIDS and the cost of further redundancies at the Agricultural and Food Research Council (AFRC).

In previous years, prior allocations have absorbed most of the extra funds available, but in the coming year there will be a significant amount left over to be divided among the research councils. Overall, the 1989–90 budget for civil science is 11 per cent greater than in the present year, which ABRC says will do much to improve morale. But ABRC also says that the allocations for the succeeding 2 years imply a reduction of 3 per cent in real terms, which

it says will mean that research councils will have to cut back on the projects they support.

With the extra funds available for next year, the Natural Environment Research Council (NERC) will spend £2.5 million on the first phase of an IRC in population biology at Imperial College, London, and £5.5 million on a centre for oceanography at Southampton. It will expand work on the biogeochemical ocean flux study (£2.7 million) and on the North Sea survey, meant to lead to a model for predicting water quality.

The Science and Engineering Research Council (SERC), the largest of those supporting natural science research, will spend £7 million next year on founding four new IRCs, in optical and laser-related science and technology, high-performance materials, polymer science and technology and process simulation, integration and control. It will also start a new programme in materials and biotechnology with £5 million of its extra allocation. SERC also has an extra £6 million for research grants, £9 million for university equipment and £0.4 million for its Cray computer facility.

The Medical Research Council (MRC) can now go ahead with an IRC in cell biology (£2 million) at Kings and University Colleges, London, in protein engineering (£2 million) at the University of Cambridge and the MRC Laboratory for Molecular Biology. It also has an extra £2.6 million for AIDS research and £2.3 million to set up an information and resource centre for mapping the human genome together with a related programme for research.

A further £2 million will allow the council to make a start on a national centre for clinical research at the Royal Postgrad-

Ozone limitations

Paris

FRENCH industry is taking steps to limit the use of products containing chlorofluorocarbons and halons. A series of conventions signed last week by representatives of government and industry are intended to reduce by 90 per cent the use of these propellants in aerosols by 1991 — two years before the target date set by the Montreal Protocol.

Beginning in 1991, aerosol cans containing chlorofluorocarbons will have to carry a written warning declaring that their use could affect the ozone layer. Manufacturers of refrigeration and air-conditioning appliances will encourage the recovery of coolants and will take measures to prevent leakage if appliances are dumped.

From the beginning of next year, manufacturers will also attach a warning to new appliances, encouraging users to collect the coolant for recycling. This will require the redesign of cooling circuits to include a drainage reservoir, as well as establishing public facilities for recovery of used coolant.

The newly signed conventions will also limit the use of fire-extinguishers containing halons. Atochem, the only French manufacturer of chlorofluorocarbons has agreed to make alternative compounds available to users.

Peter Coles

uate School at Hammersmith. There will again be more to spend on research grants (£1.5 million), studentships (£0.2 million) and equipment (£2.9 million).

The chief new projects of AFRC are an IRC in transgenic animal biology at the University of Edinburgh (£1.5 million) and new programme of research in plant molecular biology (£3.5 million), but the council will also take the lead in the government's new programme of research on agriculture and the environment (£1.5 million). The cost of restructuring the Institute for Animal Health is £3 million.

The delay in the announcement of this year's allocation of the science budget has fuelled speculation of a dispute between the prime minister, Mrs Margaret Thatcher, and the Department of Education and Science. The Labour party's education and science spokesman, Mr Jack Straw, says that the prime minister is unhappy with the department's administration of the councils, and that she wishes to move ABRC from beneath its umbrella.

But others suspect her of having intervened to contain spending on the IRCs, having advocated support for individual researchers of talent working singly or in small groups. But both the department and the Prime Minister's office deny there has been a dispute. The future of ABRC is itself being reviewed by the department; a report is due in the summer.

Christine McGourty

The UK science budget agreed for 1989–90 and planning figures for 1990–91 and 1991–92

Additions	1989–90 (£ million)	1990–91 (£ million)	1991–92 (£ million)
'Earmarked additions'	27	33	33
Research council restructuring	14	12	11
Interdisciplinary research centres	16	21	14
'Directed' programmes	23	29	34
Research grants	10	13	14
Manpower	3	5	8
Equipment	12	—	—
Other	3	4	2
Total	108	117	116
Final budget	825.6	837.6	855.8

NIH change procedures for monitoring scientific misconduct

Washington

STRUCTURAL changes are on the way in the procedures used by the National Institutes of Health (NIH) to monitor scientific misconduct. The impetus comes from a report released this week by the Institute of Medicine* (IOM) urging NIH to establish a new office to promote responsible research practices.

Although recent investigations have focused attention on misconduct in science, the IOM report looks instead at the broader question of what are appropriate standards for the conduct of research. It arises out of discussions on this issue between IOM and NIH held in 1985; a 17-member committee was appointed to write a report in 1987.

For the most part, the culture of science by its nature encourages proper conduct, says Arthur Rubenstein, chairman of medicine at the University of Chicago and chairman of the IOM committee. Tight budgets have put a variety of pressures on researchers, says Rubenstein. As well as encouraging a high rate of publication to substantiate high productivity, competition for scarce resources can tempt researchers to cut corners.

Rubenstein also cites the increasingly large size of laboratories, which makes it difficult for laboratory heads to fulfil their traditional role of monitoring the activities of their junior staff.

To combat these pressures, the IOM report suggests making explicit proper professional standards for research. Universities should set standards of conduct, provide formal instruction in research practices and create administrative positions carrying responsibility for conduct. Journal editors and professional societies are also asked to demand such behaviour from their contributors and members.

The IOM report explicitly rejects the suggestion that random data audits should be carried out in basic research. Rubenstein explains that the nature of basic research makes it nearly impossible to trace all the steps from data collection to tabulation and presentation, steps that can change as circumstances and new discoveries dictate. Such audits are more appropriate to clinical and protocol based research, where they are already used.

Meanwhile, NIH is urged not only to establish an office to encourage proper conduct, but to require by 1992 all institutions in receipt of grants to show that they have addressed the issue.

The report also suggests that NIH should limit the number of publications considered as part of a grant application to

ease the temptation to produce inappropriately voluminous *curricula vitae*.

Rubenstein says the research community is now more open to suggestions that questions of conduct should be tackled, but he expects that there will at first be resistance to the proposal that there should be special panels for addressing questions of proper conduct. He believes the initial resistance will subside when researchers see that panels can improve the research climate.

NIH deputy director William Raub says the institutes, together with the Department of Health and Human Services, are already discussing the creation of an office of scientific integrity that could fill the role recommended by the IOM report, as well

as investigating charges of misconduct. Raub says a crucial issue to be settled is where the office should be placed in the federal bureaucracy. The four possibilities being discussed are the Office of the NIH Director, the Office of the Assistant Secretary for Health, the Office of the Secretary of Health and Human Services (HHS) and the Office of the Inspector General at HHS.

The last of these options is the least favoured because the inspector-general's staff lacks scientific expertise. A final decision is bound to wait on a resolution of the political question when the position of secretary of HHS is filled. President Bush's candidate is still waiting confirmation by the US Senate.

Although the IOM report focuses on biomedical research, it recommends that the National Academy of Sciences look into the relevance of its findings for other scientific disciplines. **Joseph Palca**

US congress scuttles federal agency salary increases

Washington

THE US Congress voted last week to scuttle a 50 per cent pay increase it had been offered, and thereby perpetuated a problem with which federal agencies employing highly qualified professional staff have been struggling for some time. The National Institutes of Health (NIH) is among the most outspoken of the agencies affected.

NIH claim that their ability to recruit and retain able researchers has long been compromised by federal salary scales well below those applying at universities and in industry. The federal pay rise, which at first seemed certain to pass, would have applied to senior researchers and administrators at NIH and would, according to officials, have solved an old problem.

The proffered salary increase, recommended by an independent commission reporting every four years and endorsed by former president Ronald Reagan, would have taken effect automatically at 12:01 am on 8 February unless both the House of Representatives and the Senate had passed resolutions to reject it. The Senate did vote against the rise, but the House of Representatives had at first planned to let the deadline pass without taking action. But a huge public outcry against the proposal seems to have forced a vote — in the circumstances, inevitably negative.

After the vote, Senator Ted Stevens (Republican, Alaska) lambasted Congress for being unwilling to take the political heat for approving its own pay raise, a move that "penalizes other portions of the federal government", in particular NIH. Unlike most government laboratories,

which are run by contractors who are not bound by federal pay scales, NIH researchers are federal employees.

NIH Deputy Director William Raub says that in the past ten years the number of NIH employees at the senior executive level has decreased by 28 per cent to the present 174 people. During the same period, NIH have not been able to recruit a single person from outside the institutes. Some of the decrease is due to retirement but Raub maintains many scientists have left for higher paying positions outside government. As an example, he says that senior executive service physicians receive on average \$89,000 a year, only half the average supplemented salary paid to chairmen of clinical science departments at universities.

An independent committee of the Institute of Medicine, in a report issued last year (*Nature* 336, 701; 1988), agreed that there is a serious problem at the senior level and suggested two remedies — the establishment of an NIH foundation to finance chairs for distinguished scientists, whose salaries would then be independent of government pay scales, and the creation of a personnel demonstration project within NIH that would allow more flexible hiring and remuneration.

Raub says the committee's report is "right on the mark". Now that Congress has rejected the pay rise, NIH are looking at the report's suggestions in more detail, but cannot hope to follow them quickly because that would require that a reluctantly self-denying Congress should pass new legislation to give others the benefits of which it has been robbed.

David Swinbanks

*The Responsible Conduct of Research in the Health Sciences, National Academy Press, Washington, DC, 1989.

India's troubled space plans receive unexpected French boost

New Delhi & Paris

FRANCE last week signed bilateral agreements with India in the life sciences and agreed to help with India's troubled rocket programme and to strengthen nuclear ties between the two countries. The agreement on nuclear power would permit the French company Framatome to build two 900-MW light-water nuclear reactors in India provided that India agreed to use them for peaceful purposes only.

In the space arena, France has offered assistance for India's launch vehicle programme, which has been lagging after two successive mishaps. Hubert Curien, France's minister for research and technology, says immediate assistance can be given on the technology for clustering rocket engines, but a request for help with cryogenic motors may be delayed because of its commercial implications.

Curien says France could still provide this technology in consultation with its European partners if India agreed not to put the technology to military use and avoid any future competition with Ariane-space in the international launch business.

According to the terms of the life sciences agreement, a joint venture company will produce six different vaccines using the technology supplied by Institut Marieux, which will hold 25 per cent of the company's share capital. The vaccine plant, to be built at Gurgaon near

New Delhi, will start commercial production in 1991.

Defence cooperation was not on Mitterrand's agenda because the subject had already been discussed during the visit of the French defence minister in December 1988. France is said to have offered a \$2,000 million package for collaboration that includes a contract for the design of India's third aircraft carrier, the supply of an advanced jet trainer, air-to-air missiles and anti-tank helicopters. Avions Marcel Dassault of France has been hired as a design consultant for the light combat aircraft India is building.

The French cultural festival in India that prompted Mitterrand's visit had a marked edge of high technology, as was evident from the inaugural show—a spectacular extravaganza in Bombay's Chowpathy Bay. Thousands of Indians, in the company of Mitterrand and Prime Minister Rajiv Gandhi, watched the bay reverberate to amplified music, with giant water screens providing the backdrop for eerie images—fusions of art and science—drawn by computer-controlled laser beams reflected from huge mirrors atop barges amid a massive display of pyrotechnics that set the night on fire. According to a French diplomat, the effort was worth it if it had helped to impress on Indians that France can offer something more than wine and cheese.

K.S. Jayaraman & Peter Coles

First report on US AIDS behaviour

Washington

THE first report* solely to address the behavioural aspects of the AIDS epidemic was released last week by a committee of social scientists organized by the US National Academy of Sciences. The committee decried the dearth of information available on sexual behaviour and intravenous drug abuse, and called for more studies to find viable ways of changing people's behaviour to lower their risk of infection with HIV, the virus causing AIDS.

The committee's report is the fourth major analysis of the AIDS problem to be conducted in the United States. The Academy and the Institute of Medicine teamed up in 1986 and again last year to offer their assessment of how to counter the epidemic, and the presidential commission on the HIV epidemic issued a laundry list of about 575 specific recommendations last year. Although some of the recommendations in these reports have been implemented, more controversial steps such as

anti-discrimination legislation and random blood testing for HIV antibodies have been snarled in political battles.

The Academy committee recommends more systematic studies of human sexual practices and drug abuse patterns. Committee chairman Lincoln E. Moses from Stanford University says "AIDS is a social disease", and that not enough effort has so far been put into studying the behaviours that put people at risk and learning how best to motivate them to change. The committee also recommended anonymous blood testing of all newborns.

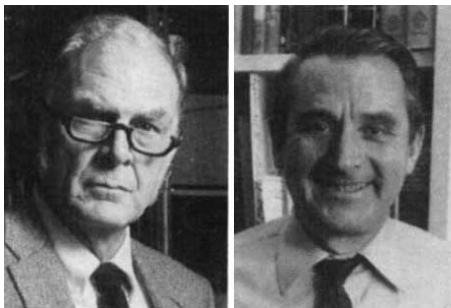
Marshall H. Becker, from the School of Public Health of the University of Michigan, says the "Just Say No" approach favoured by the Reagan Administration is not working, and that more practical approaches for reducing risk must be used. Among the committee's recommendations for encouraging behaviour changes are expanded sex education for teenagers, condom advertising on television, and free distribution of needles to drug addicts until they enter treatment programmes.

Carol Ezzell

*AIDS: Sexual Behaviour and Intravenous Drug Use, available from the National Academy Press, Washington, DC, 1989.

Japan prize awards

THIS year's Japan prize has been awarded to two US chemists working in different fields. The ¥50 million (\$400,000) awards will go to F. Sherwood Rowland, an atmospheric chemist at the University of California at Irvine, and to Elias James



Rowland (left) and Corey—Japan winners.

Corey, an organic chemist at Harvard University. They will be presented on 12 April in Tokyo.

Rowland is recognized for establishing that chlorofluorocarbons (CFCs) can destroy ozone in the stratosphere (see *Nature* 249, 810; 1974). He predicted that if CFCs were to continue to be released into the atmosphere at 1974 rates, there would be a 7–13 per cent depletion of total ozone. Corey's award is for his contributions to the synthesis of prostaglandins.

The Japan prize is awarded each year in two predetermined categories. This year's categories were medicinal science and environmental science and technology. Next year's will be for the technology of integration and for Earth sciences. J.P.

Peking man safe?

THE municipal government of Beijing (Peking) has introduced regulations to protect the site, now in the suburbs of the city, where the remains of Peking man were found. Under the new regulations all activities are prohibited that may destroy the land-form, terrain and ecological environment, including mining, tree-felling, hunting and the construction of factories that could be a source of pollution. Illegal excavation or selling or buying fossils and remains are also prohibited.

V. R.

EMBL déjà vu

LENNART Philipson, director of the European Molecular Biology Laboratory in Heidelberg, will continue in his post until 1995 rather than leave next year as originally intended.

Faced with the withdrawal of the only candidate to replace Philipson (see *Nature* 337, 397; 2 February 1989), the laboratory's council last week decided to extend Philipson's stay. His original appointment was from 1982 to 1987, which was then extended for only three years. As a condition of continuing in post beyond 1990, Philipson successfully insisted on a five-year reappointment.

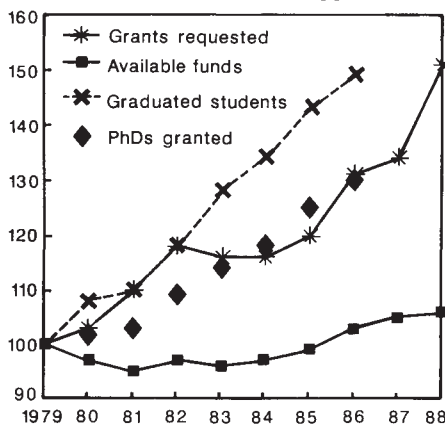
P.N.

West German agency launches early bid for more funds

Bonn

THE Deutsche Forschungsgemeinschaft (DFG) has scored an early success in its campaign for an increased budget. West German Education Minister Jürgen Möllemann (Free Democrat) announced last Friday that he will campaign for a 5 per cent annual increase for DFG, the chief source of research grants in West Germany, in each of the next 5 years.

The proposed increase is by no means a certainty, since all eleven *Länder* (states) must first approve it. On the federal level, Möllemann must secure the support of the



Grants requested and funds available from the DFG to 1988. The 1979 figures are taken as 100; no allowance is made for inflation.

rest of Chancellor Helmut Kohl's cabinet. Three-fifths of DFG's support comes from the federal government and the rest from the *Länder*. Asked about the chances of success, Möllemann said that an annual increase of 5 per cent is "realistic and achievable". The agency's budget this year is DM1,188, but the 1990 budget will not be finally approved until December.

DFG President Hubert Markl had sounded an alarm earlier last Friday when he declared that basic research support had "dramatically worsened" in 1988. He predicted grave consequences if DFG did not receive a 6.5 per cent increase in 1990.

The nub of Markl's case is that, in 1988, DFG had to reject the highest percentage of grant applications in its history: only 48.4 per cent of individual applications were funded. And for the first time, fewer than half of the *Schwerpunktprogrammen* (priority programmes) applied for could be supported (see *Nature* 336, 510; 1988).

Part of the difficulty is that research is booming at West German universities, so that there are more applications for grants than ever. Markl expects even more competition for DFG funds in the future: 40 applications have already arrived for *Sonderforschungsbereichen* (special collaborative programmes), half of them planned to begin before 1990.

There are also more students than ever crowding West German universities, which Markl says will be looking to DFG to support graduate and postdoctoral studies for the next decade. Markl says that if these people are forced out for lack of funds, their potential will be lost.

One consequence of the high rejection rate is the disaffection of DFG's (unpaid) referees, who Markl says are "fed up" with reviewing good proposals which are not supported. But he resists the suggestion that greater competition might enhance the quality of West German research: that point was reached years ago, he says, so that the applications now being turned down are of the highest

quality. Markl is concerned that West Germany will be "left behind" if it does not increase research support. He points to the planned 10 per cent increase of the budget of the US National Science Foundation and the similar increase for the corresponding body in Japan.

DFG received only a meagre increase in 1988, despite Markl's impassioned appeal in June, which is why he has felt impelled to act earlier this year, writing to Kohl and the heads of the *Länder* in January. He was visibly pleased by Möllemann's announcement last Friday.

The proposed increase is likely to be discussed at the next conference of the heads of the *Länder* on 10 March. Well-off *Länder* such as Baden-Württemberg and Bavaria are expected to take up DFG's case, while Möllemann's announcement will put political pressure on the others.

Steven Dickman

Soviet psychiatrists at odds on eve of visit by United States

London

THE campaign by Soviet psychiatrists to rejoin the World Psychiatric Association may be impeded by an open letter to the Soviet Academy of Medical Sciences by Dr Viktor Gingilis, head of the genetics laboratory of the Brain Research Institute at the All-Union Research Centre for Mental Health. The letter is a direct attack on the director of the institute at which Gingilis works, Dr Marat E. Vartanyan.

As director of the Centre for Mental Health (since December 1987), Vartanyan is in a position to influence the practice of psychiatry in the Soviet Union. The letter, first circulated at the end of last November, comes on the eve of the beginning next week of a 2-week tour of Soviet psychiatric hospitals by a group of 20 US psychiatrists and other experts.

The charges levelled against Vartanyan, who is known in the West for his categorical denials that sane but politically suspect persons were ever incarcerated in Soviet psychiatric hospitals, include many of a personal nature. But Gingilis's general complaint is that Vartanyan is seeking to shift onto others his own burden of responsibility for a practice in which he was "most active".

Reached by telephone at the institute, Gingilis acknowledged authorship of the letter, but said that he himself had not sent it abroad or to the media. Asked whether, in that case, he would prefer *Nature* not to report it, he replied "That's your affair".

Vartanyan was not available for comment, being about to leave on a business trip. His assistant, Dr Georgii Morozov, said, however, that Vartanyan knew all about the letter, and thought it a matter of little importance. "If Dr Vartan-

yan could speak to you, he would explain it all", Morozov said. An arrangement that Vartanyan would call *Nature* back at an agreed time failed to materialize.

Gingilis says that Vartanyan's election to his present post (necessary under the new democratization) was rigged, and went against the wishes of the majority of the centre's staff. His letter goes on to suggest that Vartanyan and his wife, D. D. Orlovskaya (who is at the same institute) have risen in academic distinction on the strength of the work of others, many of whom have subsequently been hounded into resignation or emigration. The letter says "I am the real author of almost all the publications and research initiatives attributed to M.E. Vartanyan in the field of medical genetics".

Gingilis claims that his own work, together with that of I. V. Shakhmatova-Pavlova, was misappropriated by Vartanyan to support his appointment as corresponding member of the Soviet Academy of Medical Sciences. He says that Vartanyan's personal power base has remained secure because few have been prepared to speak up in public, but "after twenty years of serf-like dependency on Vartanyan", he has decided to speak out "with all responsibility and with a clear understanding of the possible consequences".

It remains to be seen what will be the effect of this letter on next week's visit by the US group. The Soviet authorities maintain that all dissidents have either now been released, or are being processed for release, from psychiatric hospitals. The visitors will be able to examine patients whom they choose and to visit at short notice psychiatric hospitals which they select.

Vera Rich

Too high radon risks not helped by "penny-pinching" UK government

London

BRITISH safety limits for the concentration of radon gas in houses are too high and government proposals for financial help to those at risk are "penny-pinching and restrictive", according to the Institution of Environmental Health Officers (IEHO). The institution is the professional organization of environmental safety officers employed by local governments. Its criticisms of government policy appear in a report published two weeks ago.

The key recommendation is that the radon dose at which remedial action is considered to be necessary should be

radon above the action level. The most common way of tackling the problem is to "poke a hole in the floor of your basement, put a pipe in, run it through the ceiling and put a fan on the top", according to an EPA spokesman. This can cost between \$200 and \$1,500. The EPA is carrying out a survey to find out how many take the necessary action.

In the European Communities, no country other than Britain has any safety levels for radon concentration in the home. But the European Commission is considering recommending that member countries adopt the British standards. Sweden and Finland, the only other European countries with safety levels, set the action level at 800 Bq per m³.

The IEHO report puts the number of houses in Britain with radon levels above the present action level at 50,000, which is also twice the number estimated by NRPB. As well as the halving of the action level for which it asks, the institution says that the "priority action" concentration should be reduced from 1,000 to 800 Bq per m³ and that the "investigation" concentration should be reduced from 200 to 100 Bq per m³.

The report is especially critical of a government document giving guidance to local authorities on home improvement grants to those potentially at risk. The government expects most owners to bear the cost of remedial action themselves, but Martin Courtis, the report's author, says that if house owners are not offered a grant of at least 75 per cent of the cost of remedial work, there is little, if any, prospect that the radon problem in Britain will be tackled.

Christine McGourty



halved, from 400 to 200 becquerels per m³. This concentration was fixed two years ago by the National Radiological Protection Board (NRPB), when it was estimated that the lifetime risk of lung cancer after chronic exposure to 400 Bq per m³ would be between 2.0 and 2.5 per cent.

The NRPB now estimates the lifetime risk to be 5 per cent, and the environmental health officers say that, as a result, the action level should be halved. The NRPB is now reviewing its safety levels. But Michael O'Riordan of the board says the level will not automatically be halved: the cost of taking remedial action and the available technology will be taken into consideration before a decision is made.

Only in the United States is the action level lower than in Britain: the Environmental Protection Agency (EPA) recommends an action level of 150 Bq per m³. The agency would like this to be reduced by a factor of 10, at which point the radon concentrations indoors would be equivalent to those outdoors. But in practice it may only be possible to halve the present action level, says a spokesman. About 8 million homes, 10 per cent of the US total, are estimated to have concentrations of

Computer microcode instructions judged within copyright

Berkeley

A Californian court last week declared that microcode instructions on microprocessor chips can be protected by copyright. The decision, expected to be a landmark for microprocessor manufacturers, is likely to deter outright copying of microprocessor design.

Although computer programs were added to the US Copyright Act by Congress in 1980, the status of microcode — the set of instructions determining how a processor's components will perform a specific function — was unclear. As with any other program, a variety of instruction sets could be devised to achieve the same goal. Last week's ruling puts microcode "squarely within the definition of 'computer program'", and therefore subject to copyright protection.

The ruling also ends a 7-year dispute between Intel Corporation, of California, and NEC Ltd, of Japan. Intel claimed that NEC's 'V-series' of microprocessors infringe Intel's copyright of its 8086 and 8088 chips, used in the first generation of IBM personal computers. Although the court upheld Intel's right to copyright the microcode, it nonetheless absolved NEC of having violated that copyright, allowing both sides to claim victory in the case.

Intel and NEC entered into a general patent cross-licensing agreement in 1976 giving each access to the other's patents. The copyright dispute arose after Intel introduced the 8086 and 8088 microprocessors in 1978, and NEC began producing identical copies of the chips under the cross-licensing agreement. Intel objected, arguing that the patent-sharing allowed NEC to produce a chip of similar

function, but not to use the copyrighted microcode instructions contained in the chip. The matter was temporarily settled in 1983, when Intel licensed NEC to make the chips. Shortly afterwards, NEC developed its own 'V-series' of comparable microprocessors.

The first trial of the lawsuit was discontinued in 1986, when NEC objected that the judge owned a small amount of Intel stock. The judgement in the second trial, announced last week, declared that microcode can be protected by copyright, but found that NEC's microcode is sufficiently different from Intel's not to be a violation. Intel was also found to have forfeited its copyright by not requiring licensees to place notice of the copyright on their chips. NEC now has 11 per cent of the \$7,000 million microprocessor market, compared with Intel's 26 per cent share, but NEC officials predict that the court's vindication of its practice will improve its sales.

But Intel is also claiming victory, as the decision is likely to discourage others from copying its microprocessor designs. The loss on the 8086 and 8088 chips is financially unimportant, say company officials, as the 10-year-old chips have been replaced in personal computers by second- and third-generation microprocessors. The 8086 and 8088 chips now account for only 1 per cent of total Intel sales, says company representative Pam Pollace. She also says that the more complex microcode of the second- and third-generation 80286 and 80386 microprocessors will be much more difficult to approximate except by outright copying, which would clearly infringe Intel's copyright.

Marcia Barinaga

Oil-spill danger to nesting birds

Berkeley

A US oil cleanup crew last week reached the Antarctic Peninsula and stemmed the flow of diesel fuel from the wrecked Argentine supply ship *Bahia Paraíso*, which ran aground on 28 January near the US Palmer Base. The ship had already spilled two-thirds of its 250,000-gallon fuel supply. While the US team skimmed most of the recoverable fuel from the surrounding waters, an Argentine salvage crew prepared to remove the remaining 75,000 gallons of fuel remaining on the ship.

The biological consequences of the accident are now being evaluated. Unrecovered fuel has collected in shallow coves and inlets, where it has killed intertidal limpets, sponges and kelp, says Bernhard Lettau, National Science Foundation (NSF) programme manager for Polar Ocean Sciences, who recently arrived at the site. Lettau says the bottom-dwelling community may escape serious harm, as the water is relatively clear and free from particulate matter that could carry the fuel to the bottom.

But oil slicks less than 1 mm thick cannot be removed by skimming, so that a diesel sheen remains on much of the water in the area, according to Thomas Forhan, an NSF project manager at the site. He says that all traces of the volatile fuel should be gone within 10 days to a month, barring further leaks.

Forhan says that the ship, which is on its side in 40 feet of water, is unlikely to break up soon and that the Argentine salvage operation may take 3 to 4 weeks to recover the remaining fuel. He notes that the recovery entails the risk of further spills.

The effects of the spill on the area's krill population are unclear, according to Lettau. Deep-water animals have been less badly affected than shallow-water ones. But the spill has had a devastating impact on bird populations nesting near Palmer Base. Lettau says the breeding success of the South Polar skuas in the area "will be zero". Skuas lay two eggs in each nest, and usually only one chick survives to fledge.

But out of 53 nests inspected, only 6 had a living chick last week, and Lettau expects none of these to survive. He is unsure of the cause of death, but speculates that the chicks may be getting toxic doses of fuel in the food brought by their parents.

Lettau expects that the toll will be greatest among young birds, including penguin chicks, which fledge and take to the water in early February. Researchers at the base have begun banding chicks that have not yet left their rookeries, so as to trace their eventual fate.

Marcia Barinaga

Japan grapples with definition of death by brain death

Tokyo

THE twenty-year controversy over the definition of brain death has entered a new phase. Last week, the Japanese Diet (parliament) was presented with new legislation that will eventually decide when the absence of electrical activity in the brain (brain death) can be considered to define the death of a person. At stake is whether surgeons may legally perform life-saving heart and liver transplant operations — both of which require the donation of living organs from brain-dead people.

The legislation, which seems certain to pass, would establish a special investigatory panel on brain death and organ transplants. The panel is modelled on the US Presidential Commission on Ethical Problems in Medicine and Biomedical and Behavioral Research which, in 1981, accepted that brain death means death. But the new panel will take two to three years to reach its conclusions, which may be far too long for Japan's surgeons.

Already, a Niigata Prefecture hospital group has twice risked prosecution by performing a kidney transplant operation with an organ taken from a brain-dead patient. The first operation, last May, put the group in the odious position of having to face a legal suit for murder. Despite the risk of legal action, several other groups are eager to claim the prize of Japan's second heart transplant. The first, carried out in 1968, brought legal action and vilification for the surgeon concerned.

But now, after twenty years, a new force is propelling Japan towards acceptance of brain death. To Tokyo's embarrassment, Japanese are starting to travel abroad for transplant operations they cannot get at home. Last week, at almost the same time as the Ministry of Health and Welfare announced a new Tokyo research centre to help developing nations learn from Japan's 'advanced medical treatment' system, a fact-finding mission reported that eight Japanese patients were waiting in the Philippines for transplant operations. The mission, led by Representative Taro Nakayama (Liberal Democrat, Osaka), who has been active in promoting the new Diet legislation, also visited Australia, where it found that 22 Japanese had either received, or were waiting for, liver transplants.

With transplant organs in short supply everywhere in the world, Japanese patients in the hospitals of developing nations would seem especially incongruous. The Philippines is already considering legislative action to control visits by foreign patients.

Early last year, it seemed that Japan's

brain-death problem was about to be resolved when the Japan Medical Association's Bioethics Council released a report urging that, subject to strict conditions, brain death should be accepted as a definition of death. Surgical groups at several hospitals immediately began to file applications with their own hospital ethical boards for permission to perform transplants.

But the momentum began to wane when the Japan Federation of Lawyers' Associations announced its opposition to the redefinition of death as brain death until a full social consensus had been reached. Subsequently, in May, the Japanese Society of Psychiatry and Neurology decided to come out against the redefinition.

At nearly the same time, a University of Tokyo patients' rights group instigated legal action for murder against those responsible for the Niigata Hospital kidney transplant. The group's view is that patients' rights are not adequately protected from surgeons wishing to carry out transplant operations. Although that view finds few supporters among physicians, public criticism inhibited further transplants from brain-dead patients in 1988.

The Niigata group censured last year has already performed one kidney transplant from a brain-dead patient this year. According to relatives, the patient had expressed a wish to donate his organs in the event of his death.

Writers of *Nihonjinron*, a genre that explores supposedly unique characteristics of Japanese culture, have often asserted that the aversion to acceptance of brain death and transplant operations is related to a Japanese view that body and soul are inseparable, or to confucian concepts that the body is a gift from one's parents that cannot be given away. But the Niigata man who left instructions for his organs to be donated is reported to have been much influenced by a television programme on brain death.

The debate over definition of death has mostly subsided in the United States and Europe, with whole brain death being the commonly accepted criterion. Some have argued for extending the definition to include loss of higher brain function, but this has not gained acceptance. In most countries, solid organs may be retrieved from cadavers only with prior permission from the victim or the permission of relatives after death. But in France there is 'presumed consent', so that organs may be taken if needed over the objections of relatives unless the victim had previously made an express wish to the contrary.

Alun Anderson

Continuing discontent among Soviet scientists over elections

Moscow

CONTINUING discontent with the Soviet Academy of Science's election of nominees to the Supreme Soviet took an extraordinary turn on 2 February — a public meeting of protest outside the academy's main building in Moscow which lasted for an hour and was attended by 3,000 people.

Although public meetings are becoming a routine part of the process of democratization, the meeting outside the academy was without precedent. It is only fair to say that the academy did nothing to prevent the meeting taking place. Indeed, the ground in front of the building had been cleared of snow on the eve of the meeting, which was entirely orderly. Afterwards, the president of the academy, Guri Marchuk, received a delegation of those present at the meeting.

The issue that has exercised the scientific public is the nomination on 18 January of candidates from the academy to the Congress of People's Deputies. As a result of the voting at that extended meeting of the academy's membership, the candidates who had received most support at meetings of institute personnel were found to be missing from the final lists. Rank-and-file scientists say the outcome shows their wishes have been neglected. The five resolutions passed at the meeting on 2 February show a deep crisis of confidence in the academic community.

The most remarkable occurrence then was the contribution of Academician Vladimir Kudryavtsev, elected a vice-president of the academy only last year and put up at the meeting as the academy's spokesman. As chairman of the disputed election meeting, he could hardly have expected a hearty welcome from his critics, and there were several attempts to shout him down. He deserves praise for his determination to speak out.

"Read the slogans", he said at one point, "but I agree with most of them." "No to functionaries from science!" I also say 'No!' 'Scientists for Gorbachev and perestroika!' I also say 'Yes!' 'Let Sakharov, Sagdeev and Likhachev be our deputies!' I voted for them myself!"

Kudryavtsev went on to say that he agreed with the demand that the academy should be restructured and that a public association of scientific workers should be established. Saying that relations between the academy and its institutes should be more democratic, he asked for the meeting's help to this end.

On the outcome of the voting on 18 January, Kudryavtsev said he shared the meeting's "discontent and regret". Several days earlier, the praesidium of the academy had said that the results of the

voting had come as a bolt from the blue for everybody. Academician Marchuk said that he had also voted for Sakharov and Sagdeev.

As an observer at the plenary meeting, I had the impression that many leaders of the academy forgot to which body they were electing people, and by what principles and criteria. Counting the number of institutes backing one nominee or another

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Post-glasnost Sakharov now free to travel — en route from Italy to Canada (AP).

Sagdeev declines to stand in Kazan

Moscow

THERE has been a further escalation of the conflict within the Academy of Sciences over the nomination of candidates for the election of people's deputies. As *Nature* went to press this week, our Moscow correspondent Yuri Kanin learned that Academician Roald Sagdeev has declined to stand as a candidate for the city of Kazan, saying that he will instead "support with all my vigour" the staff of academy institutes in their "just fight against the scientific establishment".

● Kanin writes: Sagdeev has been backed by staffers at 25 research institutes of the academy, but did not make it to the list of candidates put forward by the general meeting of the academy. Instead, he was nominated by the ethnic-territorial district number eleven of the Russian Federation, which consists chiefly of the city of Kazan.

In a cable to the electoral commission of the district, Sagdeev thanks the electorate for having nominated him, hopes that they will instead nominate local followers of Gorbachev's *perestroika* but declined to stand himself.

"I feel obliged", the cable says, "to

should not have been irrelevant. Many failed to listen to Sakharov's appeal at the beginning of the meeting that there should be more confidence in the institutes' proposals. Although the voting was by secret ballot, the leadership must assume responsibility.

It is understood that the central electoral commission has said that there was "no violation of the law that would justify cancellation". But this view was not generally accepted by the commission, which agrees that the law should be further refined and made specific.

The affair is not yet over. One of the demands of the public meeting on 2 February was that the 23 candidates nominated on 18 January should now withdraw. There is also a proposal that the elections due in March should be boycotted to allow voting on a list drawn up by the institutes.

Sakharov, the front runner in the electoral process until 18 January, told me that he has now been nominated by several districts in the Moscow region and is not fully aware "who has appointed me, and where". Several other candidates disappointed at the academy elections, Academicians Dmitry Likhachev and Gavril Popov, for example, have also been nominated by other constituents. But the fate of the academy's representation in the chamber of deputies will not be known until the special conference of the academy from 18 to 22 March. **Yuri Kanin**
Novosti

support with all my vigour the staff of many institutions of the academy in their just fight against the members of the 'scientific establishment' sitting pretty at the academy's headquarters".

In an earlier interview, Academician Vladimir Kudryavtsev, the chairman of the general meeting of the academy at which the disputed nominations were made, explained that he had attended the public meeting in the academy's grounds on 2 February in a personal capacity. Saying that he had been surprised and disappointed by the outcome of the election, he denied the suggestion at the 2 February protest meeting that the list of those nominated had been drawn up by the praesidium of the academy.

Kudryavtsev also explained that while the praesidium has expressed regret at the outcome of the election, it could not have invalidated the result without flouting the procedure adopted at the outset of the election meeting. "Unless we learn to honour the laws, we will return to the times when we could only talk about democracy, while there was none".

Yuri Kanin
Novosti

Shroud irradiated with neutrons?

Sir — If the shroud of Turin is in fact the burial cloth of Christ, contrary to its recent carbon-dated age of about 670 years (*Nature* 335, 663; 1988 and 337, 611; 1989), then according to the Bible it was present at a unique physical event: the resurrection of a dead body. Unfortunately, this event is not accessible to direct scientific scrutiny, but the image on the shroud, which still cannot be duplicated, appears to be a scorch, indicating that the body radiated light and/or heat. It may also have radiated neutrons, which would have irradiated the shroud and changed some of the nuclei to different isotopes by neutron capture. In particular, some ^{14}C could have been generated from ^{13}C . If we assume that the shroud is 1,950 years old and that the neutrons were emitted thermally, then an integrated flux of 2×10^{16} neutrons cm^{-2} would have converted enough ^{13}C to ^{14}C to give an apparent carbon-dated age of 670 years.

This flux of neutrons should have other measurable consequences. The neutron irradiation would probably not have been uniform, for example, so the $^{14}\text{C}/^{13}\text{C}$ ratio

should vary in different parts of the shroud. In addition, other unstable isotopes should have been formed. Several of these isotopes have half-lives long enough that they would still be present, yet short enough that they are not found naturally.

The unstable isotopes most likely to be found in the shroud are ^{36}Cl and ^{41}Ca . The presence of either would confirm that the shroud had been irradiated with neutrons. An accurate measurement of the ratio of either ^{36}Cl to ^{35}Cl or ^{41}Ca to ^{40}Ca (see table) would test the prediction of an integrated neutron flux of 2×10^{16} neutrons cm^{-2} . This may not be possible, however, because contamination with new sources of chlorine or calcium may have occurred from washings or other sources since the irradiation took place.

THOMAS J. PHILLIPS

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HEDGES REPLIES—The processes suggested by Phillips were considered by the participating laboratories. However, for the reasons given below, the likelihood

Properties of selected parent/daughter isotopes

Parent isotope	Natural abundance (per cent)	Neutron capture cross-section (barns)	Daughter isotope	Half-life (years)	Predicted parent/daughter ratio
^{13}C	1.11	0.0009	^{14}C	5,730	1.2×10^{-10}
^{35}Cl	75.77	43	^{36}Cl	3.0×10^5	8×10^{-7}
^{40}Ca	96.94	0.40	^{41}Ca	1.0×10^5	8×10^{-9}

Predicted parent/daughter ratios (right-hand column) are for a new cloth irradiated with 2×10^{16} neutrons cm^{-2} . For $^{14}\text{C}/^{13}\text{C}$, the ratio is about 17 per cent higher than normal.

Why reprints are necessary

Sir—Ivor Smith (*Nature* 336, 708; 1988) complains of receiving reprint requests for a short paper and claims that “the cost of sending for this reprint and the time involved are appreciably greater than walking to the library and making a single photocopy for one’s own use”. He further suggests that photocopying articles, rather than sending reprint requests, is the “sensible approach”.

Perhaps he is correct, if one reads only one short article each week. But in many scientific fields, including my own, one comes across 20–30 articles a week. The cost and time spent walking to the library, finding the articles and photocopying 30 articles is significantly greater than the cost and time required to fill out reprint requests. The volume of research articles is such that only with the time-efficient method of perusing *Current Contents* and requesting reprints of significant articles do I have a hope of having the time to actually read the articles. Thus, for many

scientists, the reprints are requested on the basis of the title of the article, which is why it seems to Smith that the requesters have not yet read the article (and apparently will not read the article, considering that they do not receive the reprint).

If it is true that the “European reprint is a disappearing commodity”, then perhaps the numbers of scientists reading European articles will be reduced, and European scientists themselves will be even more hard-pressed to keep up with the literature. This may be another funding-related factor which contributes to the decrease in scientific productivity in Britain, lamented in *Nature* over the past two years. Reprints may be expensive, but I believe that they remain the most time-efficient method of article acquisition, and will remain so until the development of ‘electronic’ journals.

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that they influenced the date in the way proposed is in my view so exceedingly remote that it beggars scientific credulity.

(1) No plausible physical mechanism has been proposed to explain how the resurrection was accompanied by a significant neutron flux. If a supernatural explanation is to be proposed, it seems pointless to make any scientific measurement on the shroud at all.

(2) Assuming a ‘scientific’ (but not yet articulated) explanation for the neutron flux, it is an amazing coincidence that the neutron dose should be so exactly appropriate to give the most likely date on historical grounds. (Arguably a total of 10^{28} neutrons (the number in a human body) would be available. Using Phillips’ figures, this would be sufficient to impart a date of 100,000 years into the future. To produce a date within 100 years of the first recorded history of the shroud implies that the dose has been ‘fine-tuned’ to better than one part in a hundred million.)

(3) In fact, the dose proposed by Phillips is much too high, as he has not included the neutron capture by nitrogen in the cloth. A not untypical N content in linen is 1,000 p.p.m., for which a thermal neutron flux of $2 \times 10^{13} \text{ cm}^{-2}$ (that is, 1,000 times less) would be appropriate. This does not change the basic argument, but changes the chemical implications (even without recoil effects). The three dating laboratories used different types of chemical pre-treatments, (for example at Oxford we purified the cellulose), yet obtained equivalent results. This shows that any ^{14}C formed by neutron irradiation behaves chemically in the same way as the original ^{14}C . This is inherently unlikely because the original nitrogen is in a chemically quite different environment.

(4) As Phillips comments, one might expect that the ‘irradiation’ would be non-uniform. The three samples were contiguous, but at least on a local scale of 10 mm, any such variation was less than 1 per cent.

The lower neutron flux would reduce the conversion of ^{35}Cl to ^{36}Cl , but should still give a ratio significantly above background (if there has been no loss or gain of Cl since). Electron spin resonance signals from the ‘irradiation’ might also persist. However, such measurements are unlikely to confirm the presence or absence of neutron irradiation with absolute certainty. This, I fear, will not be achieved in a finite number of tests. If we accept a scientific result, we must exercise a critical notion of the probabilities involved. If we demand absolute certainty, we shall have to rely on faith.

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Supernova springs new surprise

What seems to be the pulsating star left by the supernova whose explosion was observed two years ago has surprised everybody and could, if confirmed, stand theory on its head.

ASTRONOMERS have been growing a little anxious in the past few months, wondering when the pulsar left by the explosion of supernova 1987A would emerge from the cooling remnant. The longer they waited, the more they suspected that the pulsar would be feeble and undistinguished. Instead, if observations announced last week stand up to scrutiny, astronomers are faced with three surprises — and a raft of theoretical puzzles.

The bare facts, given in IAU Circular No. 4735 by John Middleditch of Los Alamos National Laboratory, Carl Pennypacker of Lawrence Berkeley Laboratory and many others, are that optical variations between magnitudes 18 and 19 were detected during a 7-hour observing period on 18 January with the 4-m telescope at Cerro Tololo Observatory in Chile. From these observations comes a pulsar with a period of half a millisecond, making it the fastest known. And there is also an 8-hour variation suggesting that the pulsar is a member of a binary system.

The three surprises are these. How can a new-born pulsar have a sub-millisecond period, when it has been thought for years that pulsars are born rotating perhaps ten times a second? Why is the pulsar's magnetic field so small, which it must be because luminosity goes as the square of the field strength and the fourth power of the rotation rate, and because the pulsar is certainly no brighter than the much more slowly rotating Crab pulsar? And how has it acquired a companion which, had it been present at the time of explosion, would have been sitting in the outer layers of the giant progenitor star?

Most pulsars have periods of some modest fraction of a second; the few with millisecond periods belong (with a couple of exceptions) to binary systems. So, the story goes, all pulsars are born rotating slowly, and spin down as they age. But a pulsar in a close binary can accrete mass from its companion, and be spun up to a millisecond period as mass spirals in, conserving angular momentum. The handful of isolated millisecond pulsars are supposed somehow to have lost their companions, perhaps through encounters with other stars or, as in the celebrated case of PSR1957+20, by blasting them away by radiation.

This tidy picture absolves astrophysicists who calculate the dynamics of supernovae explosions from the immense trouble of dealing with rotation, but the

1968,629-Hz periodicity of the new pulsar changes that picture. Its rotational energy is about the same as its gravitational binding energy, which means that it is on the point of being physically flung apart. (For some theoretical nuclear equations of state, it ought to fly apart, which is another problem.) Moreover, the energy of the explosion was only a small fraction of that now tied up in rotation, which means that refinements of the distribution of angular momentum in the progenitor yield order-of-magnitude effects in the explosion dynamics.

For Stan Woosley of Lick Observatory, who has exploded many stars by computer, the problem is that no one knows the distribution of angular momentum in a giant star, or how to calculate angular momentum transport through a convective turbulent medium evolving on a millisecond timescale.

The angular momentum of a giant star, conserved through collapse to a neutron star, is enough to create a millisecond pulsar. But hoping to follow the collapse in detail, and to make useful predictions about the outcome, is close to impossible.

Woosley is understandably waiting to see whether the observations are confirmed before he attempts to explain the new pulsar, and is not averse to models in which the pulsar was born slow two years ago, but has been spun up by accretion since then. But others, such as Andrew Fabian, of the Cambridge Institute for Astronomy, are sceptical. Although dumping about half a solar mass of material onto a fledgling neutron star could spin it up to a millisecond period, the energy generated would be far above the Eddington limit (meaning that radiation pressure would be enough to repel the infalling material).

The Eddington limit can be evaded, for example by supposing gas to accrete at the equator and radiation to emerge from the poles, but this requires special pleading. And the extra gas must come from somewhere: if the supernova explosion has to be such that the core collapses, the atmosphere is blown off yet a little gas is left stranded for subsequent accretion, the pleading becomes even more special.

Like its rapid rotation, the low magnetic field of the new pulsar will probably just have to be accepted for what it is. The elementary assumption that a pulsar obtains its luminosity from electrons accelerated by the rotating magnetic field

leads to a simple relation between brightness, magnetic field and rotation rate. The dipole field strength of the new pulsar can be no more than about 10^9 gauss, whereas 10^{12} gauss is what astrophysicists would have guessed.

If the rapid rotation and low field are odd, the apparent 8-hour orbital period is bizarre to the point of incredibility. The companion apparently has the mass of Jupiter and an orbital radius putting it inside what would have been the outer hydrogen layer of the progenitor.

While it is on the fringes of possibility that a condensed object might have lived for a little while in the cool atmosphere of a giant star, it is quite impossible that any such object could have survived the explosion. Some unusual hypotheses come to mind: if the pulsar was spun up by recent accretion, then the companion could be a blob accreting material; if the core was rotating very fast as it collapsed, then it might have passed through a "dumb-bell" phase and fractured into two pieces.

The recent case of PSR1957+20, in which radiation from a pulsar is presumed to be eroding a nearby companion, suggests to Edmund van den Heuvel of Amsterdam University a similar but more extreme history for the pulsar in SN1987A. Because of its very rapid rotation, the newly formed pulsar may have the power to boil away a companion of one solar mass in a matter only of years, rather than the millions of years taken by PSR1957+20. To do this, a much stronger magnetic field than the simple-minded limit allows is needed but because the system is evidently evolving on a timescale of years, even months, the usual arguments may be quite wrong.

The reality of the 8-hour periodicity is certain to be questioned. But Pennypacker and his colleagues are the best in this business, and they spent several weeks analysing their data because they too were taken aback by what they found. New observations are scheduled two weeks from now. The new pulsar may be a freak but van den Heuvel at least seems prepared for a wholesale revision of ideas: the notion that pulsars are born slow may turn out to be an unfounded guess, an unwarranted extrapolation to the moment of birth from 1000-year old objects such as the Crab pulsar. The infancy of the new pulsar will be followed minutely, and will put adulthood and old age in a new light.

David Lindley

Retroviral proteinases

A second front against AIDS

Tom Blundell and Laurence Pearl

Of all the main infectious diseases in man, those caused by viruses are the greatest challenge to the development of effective drugs. Retroviruses such as human immunodeficiency virus (HIV) code for three enzymes of their own: reverse transcriptase, integrase and proteinase. Attempts to develop drugs have so far focused on the inhibition of the viral reverse transcriptase. This enzyme is the target of AZT, the most effective anti-HIV drug in clinical use. Although screening compounds for antiviral activity could turn up useful drugs, it is knowledge of the three-dimensional structure of the virally encoded enzymes that should allow *de novo* design of compounds with high specificity and low toxicity. The basic data for receptor-based design of a new generation of anti-AIDS drugs are provided by two new X-ray analyses of retroviral proteinases (or proteases). Last week, Wlodawer and colleagues reported the structure of Rous sarcoma virus (RSV) proteinase, and on page 615 of this issue², Navia and co-workers report the structure of the enzyme of HIV-1 itself.

Tang *et al.*³ in 1973 observed an unusual sequence of three amino acids, Asp-Thr-Gly, that occurs at both of the two aspartate residues at the active site of pepsin. Subsequently it was discovered⁴ that aspartic proteinases such as pepsin have a bilobal structure with a well-defined cleft. A pseudo dyad relates two similar domains with Asp-Thr-Gly sequences occupying topologically equivalent positions and hydrogen-bonded at the centre of an extended active-site cleft. Both studies^{3,4} suggested these proteins evolved from an ancestral dimeric enzyme. Toh *et al.*⁵ found clues for a present-day relative of this ancestral aspartic proteinase by identifying a highly conserved Asp-Thr-Gly sequence in retroviral sequences in regions thought to encode the proteinase responsible for maturation of the virus. Alignment of consensus sequences for the retroviral proteinases with the sequences of the two lobes of pepsin-like enzymes^{6,7}, together with the finding that there is weak inhibition of retroviral proteinases by pepstatin⁸, indicated that the retroviral enzyme would be dimeric and have a pepsin-like fold. These observations suggested that retroviral inhibitors, perhaps useful for the treatment of AIDS, might be achieved using the technology previously developed for the design of antihypertensive inhibitors of renin⁹, an aspartic proteinase related to pepsin.

The newly described crystal structures of the two retroviral proteinases — RSV

at high resolution¹ and HIV-1 at medium resolution² — confirm the prediction of a dimeric structure with a fold similar to other aspartic proteinases⁶. Each enzyme comprises two subunits, each containing a mixed β -pleated sheet of two intertwined motifs of four strands (a, b, c, d and a', b', c', d') related by a pseudo dyad as shown in Fig. 1. The active-site Asp-Thr-Gly or Asp-Ser-Gly sequence occurs on the wide loop (c, d) which is crossed by strand d' to give a PSI-like structure. Although only the RSV, and not the HIV-1 structure is reported at resolution high enough reliably to indicate the side-chain conformations, it appears that both

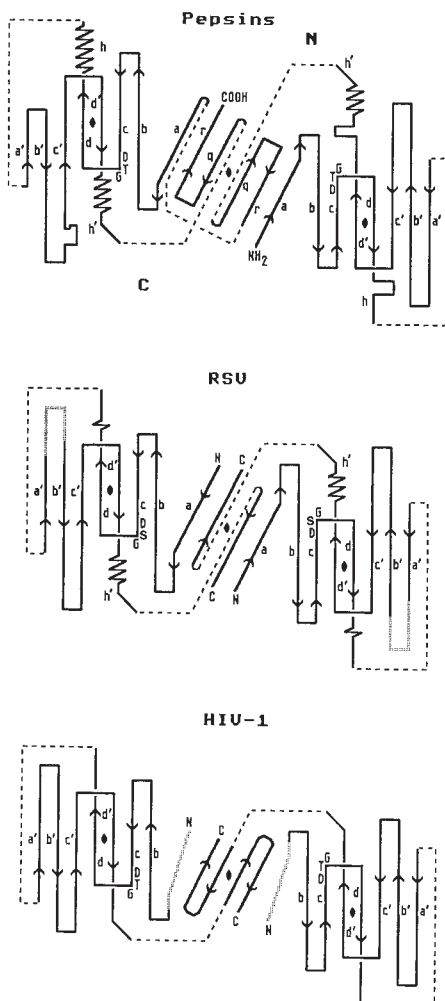


Fig. 1 Arrangements of β -strands and α -helices in pepsins and in the retroviral proteinases of RSV and HIV. In the three-dimensional structures the sequences DT(S)G on strands c are hydrogen-bonded together at the centre of the active-site cleft around the dyad (see Fig. 2). Regions that are unclear in the electron-density maps are shown in grey; dashed lines indicate connections that are short in three dimensions.

enzymes have the characteristic aspartates hydrogen-bonded together at the centre of the active-site cleft^{1,2}. The serines or threonines stabilize the symmetrical dimer in an exactly analogous way to pepsin by forming a buried 'fireman's grip' between the subunits¹⁰.

The variations in three-dimensional arrangements of the carboxy-terminal strands and the adjacent amino-terminal strand shown in Fig. 1 reflect the shorter sequences of the retroviral proteinases when compared with the pepsins. Nevertheless, it is surprising that there are differences between the two viral enzymes, including the absence in the HIV enzyme both of a helix in a relatively well-conserved region, h', and of a clearly defined amino strand. Although these strands are involved in subunit interactions within the dimer, the relative positions of the subunits are well conserved between retroviral proteinases and pepsins. Furthermore, the variations at the chain termini occur mainly on the opposite side to the cleft, and therefore will not greatly affect the pockets that define enzyme catalytic activity or substrate specificity.

The extended hairpin a', b', which forms the 'flap', also varies greatly, and there is no equivalent of the invariant Tyr 75 of pepsins that may stabilize the transition state⁹. In fact, the end of the flap of RSV proteinase cannot be seen in the electron-density map even at 2 Å resolution¹, indicating high mobility. By contrast, the two flaps of the HIV proteinase are hydrogen-bonded to neighbouring molecules², as in penicillopepsin¹¹, and so, although they are clearly visible, their position may not be relevant to the enzyme in solution. In any event, it seems that the function of one or both flaps in the retroviral enzymes is to interact with the substrate and to exclude water from the substrate-binding site.

Comparisons and analogies between the structures of retroviral and aspartic proteinases should be useful for the design of HIV proteinase inhibitors. Although the two subunits are completely symmetric in retroviral proteinases, the substrate is asymmetric. Consequently, identical regions in the two subunits must interact with different regions of the substrate on either side of the scissile bond; this may induce asymmetry in the retroviral proteinase itself. By analogy with the pepsins, the substrate binding appears to be achieved by exploiting a weak pseudo symmetry in the substrate⁹. Thus, side-chains of P₁ and P₁' probably bind in identical hydrophobic pockets (Fig. 2). The chain is likely to be positioned by hydrogen bonds between the main-chain amino functions of P₁ and P₂' and the carbonyls of Gly 27 of each subunit of HIV proteinase. A further main-chain interaction at P₃ with the main-chain amide and side-chain oxygen of Thr 219 in

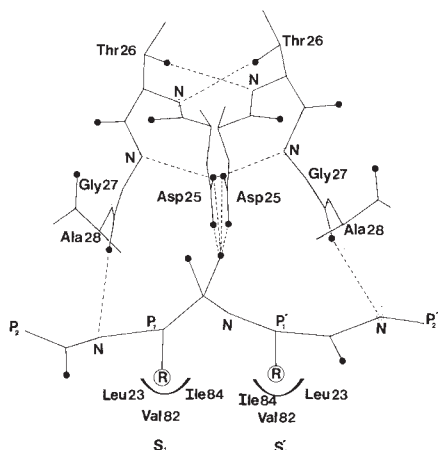


Fig. 2 Arrangements of the active-site residues Asp-Thr-Gly-Ala at the active site of retroviral proteinases, together with a model of the transition state of the substrate based on X-ray studies of analogous pepsins. Dashed lines indicate possible hydrogen bonds. The scissile bond lies between P₁ and P₁'.

pepsins^{7,12,13} may be replaced by main-chain amide and side-chain oxygen of Asp 29 in HIV proteinase. The almost invariant Arg 87, which has no analogue in pepsins, appears to be ion-paired to this highly conserved aspartate. Most excitingly, the perfect symmetry of the retroviral proteinases implies that their inhibitors could also have perfect 2-fold symmetry, giving a considerable gain in affinity for the inhibitor relative to the substrate transition state.

What do the crystal structures tell us about the biological function of the proteinase in viral assembly and maturation? The molecules observed in the crystals are not the initially translated forms of the enzymes, but derive from larger precursors. In RSV, the proteinase is synthesized as part of the Gag polyprotein, whereas in HIV it is encoded as part of the Gag-Pol fusion polyprotein. The Gag and Gag-Pol precursors are the native substrates for the viral proteinases. As there is no evidence for involvement of cellular enzymes in the proteolytic processing of Gag and Gag-Pol precursors — in contrast to the processing of the Env precursors — the proteinase must be responsible for the cleavage of its own precursor.

Because the dimer is the active form of the enzyme, the question arises as to whether the precursor cleaves itself or whether it is cleaved by another dimer. Navia *et al.*² in this issue suggest that in the HIV proteinase the disordered amino-terminal strands are sufficiently flexible to fold into the active-site cleft so that they can be cleaved by an intramolecular reaction, similar to the mechanism that occurs in pepsinogen¹⁴. In the RSV structure, however, this strand appears to be well ordered. It is unlikely that the carboxy-terminal strand of either viral enzyme could be cleared intramolecularly without disrupting the integrity of the dimer.

It is the precursor that forms the initial budding 'immature' viral particles, and careful spatial and temporal control of the proteolysis must be important for the production of mature infectious virions. Navia *et al.*² suggest that proteinase dimers will be active in a precursor form before budding, but will not cause premature cleavage of membrane-associated Gag precursors because of effective dilution in the large volume of the cell.

There are other mechanisms for control of the initiation of the proteinase cleavage. Aspartic proteinases as a class are optimally active at acidic pH and retroviral proteinases behave similarly. Mammalian aspartic proteinases such as pepsin are synthesized as inactive zymogens and are activated by a drop in pH¹⁵. A similar mechanism may operate in retroviruses by progressive acidification after budding or by budding into exocytotic vesicles.

Evidence from other retroviruses¹⁶ suggests that proteinase activity might also be dependent upon the correct stoichiometry of the Gag and Gag-Pol precursors and their correct assembly in the icosahedral capsid of the budding virion. It is possible that the precursors occupy special

positions in the icosahedral symmetry of the immature virus particle and that the molecular 2-fold axes that generate the active proteinase dimers coincide with the 2-fold axes of the icosahedral immature capsid. □

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Microelectronics

Feedback cooling of currents

Sean Washburn

JUST as feedback can be used to correct errors in the output voltages of amplifiers, it can also be used to remove noise from the current through a resistor — including the 'Johnson/Nyquist' noise that results from the thermal motion of electrons¹. Such a feedback amplifier behaves as a refrigerator cooling the electrons in a resistor connected to it. This principle has been recognized since the 1940s (ref. 1), but largely ignored because the cooling power available from such refrigerators is minuscule. John Price points out in a new paper² that the method might be practical for cooling the currents in (but not necessarily the materials of) the microscopic circuits that are typical of modern electrical engineering and recent studies in transport physics. Because of an analogy to the method used in collimating particle

beams in high energy physics experiments, he names the process 'stochastic cooling'.

The thermal energy associated with a non-zero temperature in materials is distributed not only among the available vibrational motions of the lattice, but also in random agitations of the electrons. These give a finite limit to the precision with which currents can be detected, and appear as noise limiting the sensitivity of any electronic circuit. A brute-force method to reduce the noise is to cool the whole electronic device, say, in liquid helium. But the method discussed by Price represents a more subtle approach to cooling the electrons alone.

In very small, thermally isolated resistors (typical metal wires with volumes less than about 1 μm³), Price's feedback refrigerator might be sufficiently powerful to lower the electron temperature in the wire from $\approx 10^{-2}$ K (a temperature within reach of conventional refrigerators) to less than 10⁻⁶ K. If accomplished, this would mark a lowering of electron temperatures by more than an order of magnitude below the current record, which is about 10 μK obtained by nuclear demagnetization (cooling of the nuclear-spin temperature)^{3,4}.

The cooling circuit (Fig. 1) uses an 'active resistor' R_f to absorb energy from a passive resistor R . The noise current from

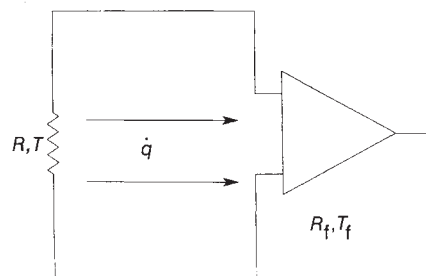


Fig. 1 Circuit diagram of the essential elements of the feedback refrigerator.

the resistance R at temperature T is then cancelled by the feedback loop just as other error signals would be cancelled in a more conventional application. A current of heat flows from R to R_i and is dissipated there. Under operating conditions ($T \gg T_i$), the heat current or cooling power is $\dot{q} = kT\Delta f$, where kT is the thermal energy of the electrons in R and Δf is the bandwidth of frequencies over which R and R_i communicate. Practically speaking, Δf cannot be much greater than 10 gigahertz (GHz), which means that $\dot{q}$ will not exceed 10^{-12} watts (W), and it is likely to be much smaller. The typical cooling powers from dilution refrigeration or demagnetization are orders of magnitude greater (in the microwatt range) but at concomitantly higher base temperatures³.

To obtain the lowest electron temperatures, one must employ an amplifier with

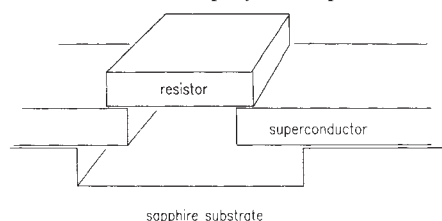
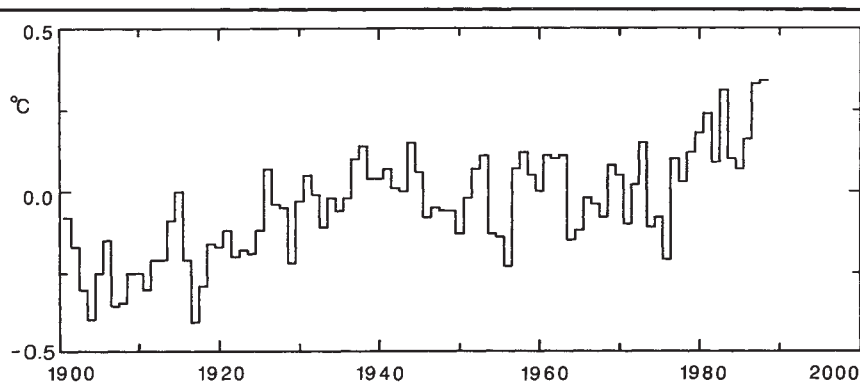


Fig. 2 A possible realization of the refrigerator that thermally isolates the resistor.

very low intrinsic noise in making R_i . The best candidate is the superconducting quantum interference device (SQUID) comprising a loop of superconducting material broken at two points by tunnel barriers, especially the double-interferometer or direct-current SQUID. The amplifier works by phase-locking the wavefunctions of 'Cooper' pairs of electrons (which are the basis of superconductivity) across the tunnel barriers. In this case the feedback circuit (operated typically at 4 K) is likely to limit the base effective noise temperature of R_i , but this is quite small in some amplifiers — as low as $T_i \leq 1 \mu\text{K}$ (ref. 5) — and the fundamental limit is lower. The output noise from the feedback amplifier could be coupled (very weakly) to another SQUID and be read as an indicator of the electron temperature in the wire. The usefulness of the technique depends on surmounting the two design problems (which Price acknowledges) of reducing the heat flow into the sample below the cooling power of the refrigerator and of making a suitable coupling circuit between the passive resistor and R_i .

To imbed a thermally isolated resistor into the circuit, Price suggests the use of superconducting contacts. This might be accomplished as in Fig. 2 where the metal wire forms a bridge between the superconductors, which in turn are connected to the rest of the feedback refrigerator. The sapphire substrate is a strong enough thermal conductor to allow a conventional refrigerator to lower the temperature of



LAST year was the warmest year since records began. Global temperature records analysed at the UK Meteorological Office and the University of East Anglia show that the global average temperature for 1988 was 0.34°C above the long-term (1950–1979) average and that eight of the nine warmest years this century have occurred in the 1980s. Whether this apparent warming trend is a consequence of the 'greenhouse effect' is moot; as variations ranging from the daily changes in weather to the long-term glaciations show, global temperatures fluctuate considerably owing to natural causes. But the observed warming is consistent with model predictions of the greenhouse effect.
Philippa Lloyd

the whole circuit into the millikelvin range. The feedback refrigerator would then have to surmount the heat leaks shown in Fig. 3. The greatest heat leak into the wire is through the thermal conductance of the superconductors, and at temperatures far below the 'energy gap' of the superconductor this leak is typically³ (although not always⁶) quite small. At such temperatures, essentially all of the electrons in the superconductor are bound up in Cooper pairs that carry no heat at all.

The only remaining thermal conductance is through vibrations of the lattice (phonons) of the superconductor to the lattice of the wire, and then into the bath of normal electrons in the wire. Again the low temperatures reduce this drastically; the thermal conductivity of the phonons in the lattice falls as the cube of the temperature. The coupling of the energy from the

phonons to the electrons in the metal also falls as a power law in the temperature. So overall, at very low temperatures, the electrons are likely to be largely decoupled from the lattice temperature (see Fig. 3).

The problem of the coupling between the passive and active resistors is thorny. It must limit the bandwidth of frequencies between which R and R_i communicate to the range in which R_i exhibits low noise and is capable of cooling, but it must dissipate no energy. Any dissipation in the coupling is noise (increased temperature) that can be dissipated in R . The coupling must therefore comprise purely reactive elements linked by superconducting wires. This in turn causes further complications. The use of superconducting contacts requires that a magnetic field be applied to quench any proximity-effect superconductivity in the resistor. And the magnetic flux in the superconducting elements can lead to dissipation providing a further heat leak.

Assuming that the difficulties in the connecting circuitry can be overcome, Price shows that for reasonable values of resistor size and phonon temperature a conservatively specified direct-current SQUID (noise levels far in excess of the best yet obtained⁷) should be able to cool the electrons in, say, a thin copper film to less than 1 mK. For extreme design criteria, the base temperature might be orders of magnitude lower. □

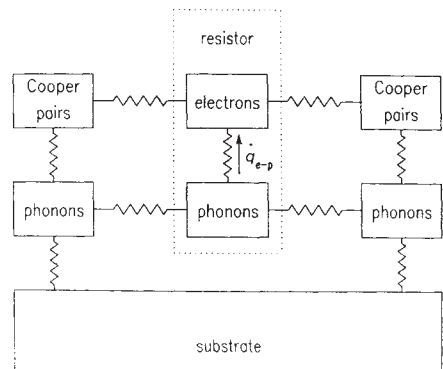


Fig. 3 The heat conduction paths into the resistor in Fig. 2. The circuit elements indicate thermal baths and thermal conductances among them. Measurements⁷ indicate that the coupling between the electrons and the lattices is poor even at temperatures ≥ 10 mK. Typically the heat leak from the lattice to the electrons is $\dot{q} = \alpha VT^2$ (V , volume). Because the coupling strength α is rather small^{7,8} and the exponent z is large (3–5; ref. 8), this heat leak should be small, so that cooling of the electrons separately from the lattice should be possible.

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Multiple sclerosis

Relationship to a retrovirus?

Byron H. Waksman

E. PREMKUMAR Reddy and colleagues at the Wistar Institute in Philadelphia and the University of Lund in Sweden, in the 27 January issue of *Science*¹, describe the identification of DNA sequences corresponding to human T-lymphotropic virus (HTLV-I) in the peripheral blood mononuclear cells of six Swedish multiple sclerosis patients. They also describe their failure to find similar sequences in all but one of twenty healthy controls (ten Swedish and ten North American). Some but not all of the patients are reported to have antibodies against the virus in their blood.

This report is an extension of studies published by the same group 3 years ago² in collaboration with Robert Gallo's group at the National Cancer Institute. The new work¹ involves the use of the powerful polymerase chain reaction (PCR), which detects and amplifies low concentrations of viral material, allowing molecular cloning and sequencing of the amplified DNA. The report has, not surprisingly, caused a flurry of excitement in the media, because it suggests a relationship between a retrovirus related to HTLV-I and multiple sclerosis. It does not imply that the retrovirus is the cause of the disease, and the authors do not claim this. But it is possible that these findings will stimulate attempts to develop preventive viral vaccines or therapeutic investigations with drugs which arrest retroviral replication, such as those being developed for the distantly related AIDS virus.

Reddy and collaborators used typical multiple sclerosis patients in their new study¹, many of whom had exacerbating-remitting disease. These were clearly distinct from cases of the slowly progressive, multiple sclerosis-like disease tropical spastic paraparesis, or HTLV-I-associated myelopathy, which is widely distributed in Africa, the Caribbean area and southern Japan. This disease is clearly associated with, and probably caused by, HTLV-I, and clear-cut viral isolation has now been reported by several groups^{3,4}.

DNA sequences corresponding to both the *gag* and *env* regions of the HTLV-I genome were identified in peripheral blood mononuclear cells from all six multiple sclerosis patients studied by Reddy and collaborators¹. The results seem persuasive because of the sharp distinction between patients and controls; the concordance of the findings with two different retroviral genes; and the sequencing data provided. At least one other group of investigators, in a study which will shortly appear⁵, has succeeded in demonstrating HTLV-I-like DNA sequences

in the *pol* and *env* regions in some multiple sclerosis patients but not in normal controls. But when this group used a *gag* primer similar to that used by Reddy *et al.*, all of a series of 21 multiple sclerosis patients, 35 normal controls and 10 cord blood samples gave positive results.

The most interesting possibility raised by the results from both groups^{1,5} is that an exogenous retrovirus related to HTLV-I may have a direct causative role in multiple sclerosis. The failure of most laboratories to find antibodies reactive with this virus, and the discrepant antibody finding of Reddy *et al.*¹, speak against this. Another possibility is that Reddy *et al.* are looking at 'endogenous' retroviral DNA sequences, which are widespread in human and other mammalian germline DNA⁶. For some viruses, the complete structure, including *gag*, *pol* and *env* sequences, is often maintained; intact infectious virus is usually not found. At least five known families of retroviruses have been demonstrated in the normal human genome. When supersensitive techniques like the PCR are used, retroviruses resembling HTLV-I can be found in many individuals⁶. But if endogenous retroviral sequences account for the multiple sclerosis results of Reddy *et al.*¹, why were 19 of the 20 controls negative? Results obtained with the PCR technique vary greatly from day to day, and Reddy *et al.*¹ do not report how often they carried out the tests in the multiple sclerosis samples and the controls or even if they ran the multiple sclerosis and control samples concurrently. There is therefore some uncertainty whether the possibility of endogenous sequences is excluded.

Another spectre haunts those who use the PCR technique — the possibility that the test system is contaminated with retroviruses already present in the laboratory. Some take extreme precautions to deal with this possibility, carrying out the PCR in a different laboratory or building than that in which cell separations are done. Contamination can be excluded when the sequences found differ at many positions from all possible contaminants. However, the sequences described by Reddy and colleagues in the multiple sclerosis samples differ only slightly from the comparable sequences of the HTLV-I (derived from patients with tropical spastic paraparesis) studied earlier in the same laboratory. Because the PCR is not an error-free technique, this may be a source of concern.

It is regrettable that the investigators failed to test their techniques on blood cells from patients with diseases related to

multiple sclerosis, such as rheumatoid arthritis or juvenile diabetes, or even on mitogen-stimulated normal cells, as the circulating monocytes and lymphocytes of both main subsets are markedly activated in multiple sclerosis, as well as in these other conditions⁷. Cell activation by, for example, phorbol esters results in enhanced retroviral gene expression⁸. Although any possible effect of such activation on the PCR staining of DNA is conjectural, it would be reassuring to know that tests for HTLV-I on activated blood mononuclear cells of such controls were consistently negative.

The pathogenesis of the characteristic inflammatory destructive lesions of multiple sclerosis, rheumatoid arthritis, insulin-dependent diabetes, chronic thyroiditis and other similar diseases is explained by many as an immunological reaction to tissue antigen triggered by viral infection (measles, rubella, Epstein-Barr virus) in early life in genetically predisposed individuals⁹. This view is based on epidemiological evidence and the study of autoimmune animal models (see my earlier News and Views article¹⁰), and receives strong support from recent studies of genes associated with susceptibility to multiple sclerosis and of those governing recognition of myelin antigens by T cells. Perhaps an autoimmune reaction can trigger the local expression of exogenous or endogenous retroviral genes, which make a secondary contribution to lesion pathogenesis, but this remains little more than a speculation.

To conclude on a sobering note, one should recall that more than 20 viruses have been incriminated as the possible cause of multiple sclerosis since 1946¹¹. In twelve instances, the presumed causative virus was actually isolated from multiple sclerosis patients. Yet none of these candidates has stood the test of time. This underlines the need for further studies in other laboratories to confirm the findings of Reddy *et al.*¹ and for control studies in other neurological and autoimmune diseases. Putting these cautions to one side for a moment, the new findings are among the most interesting to be published in recent years in this difficult field. □

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Structure of galaxies

Origin of the Hubble sequence

M.G. Edmunds

MORE than 60 years have elapsed since Edwin Hubble devised his elegant and concise classification system for galaxies (see figure), but we still do not have a good theory of the mechanisms by which the various types arise. A possible pointer to the correct explanation comes from some recent, albeit rather incomplete, dynamical calculations of Lake and Carlberg¹ performed on a Cray X-MP supercomputer. In their simulations, which incorporate dark matter (a common ingredient in cosmological theories²), the authors find that the final form of the galaxies depends strongly on the initial spread of velocities in the pre-galactic distribution of dark matter.

Nearly all galaxies have a spheroidal component — a fairly smooth, three-dimensional distribution of old stars. But spiral galaxies (both barred and unbarred) also show a characteristic thin disk of stars, containing gas and dust. The basic questions are what determines whether a galaxy has a disk, and whether the spheroidal components of spirals are really equivalent to elliptical galaxies.

Although there have been alternative proposals, it is usually assumed that elliptical and spiral galaxies formed at about the same time, and that there is no evolution from one type to another. It has long been realized that, if this is true, the critical process determining the basic type of a galaxy must be the time taken for gas to turn into stars compared with the time required for the initial gas cloud to separate out from the primordial matter during the early expansion of the Universe and collapse under its own gravity. If the gas forms into stars quickly enough, the kinetic energy of the collapse is preserved in the stellar motions. Because the stars rarely interact with each other, a spheroidal or ellipsoidal ball of stars results. If the star formation is slower, then much of the gas can collapse into a disk, dissipating its kinetic energy by collisional shocking of the gas before it is converted into stars. This leads to a disk galaxy, with continuing slow star formation in the disk and a modest spheroidal component comprising the stars which did form during the collapse.

The question then becomes one of deciding what it is that regulates the rate of star formation (local gravitational collapse) relative to the rate of the global dynamical gravitational collapse of the whole galaxy. Thus it has been suggested³ that star formation is promoted by increased local gas density and inhibited by increased angular momentum per unit mass. The latter suggestion is attractive

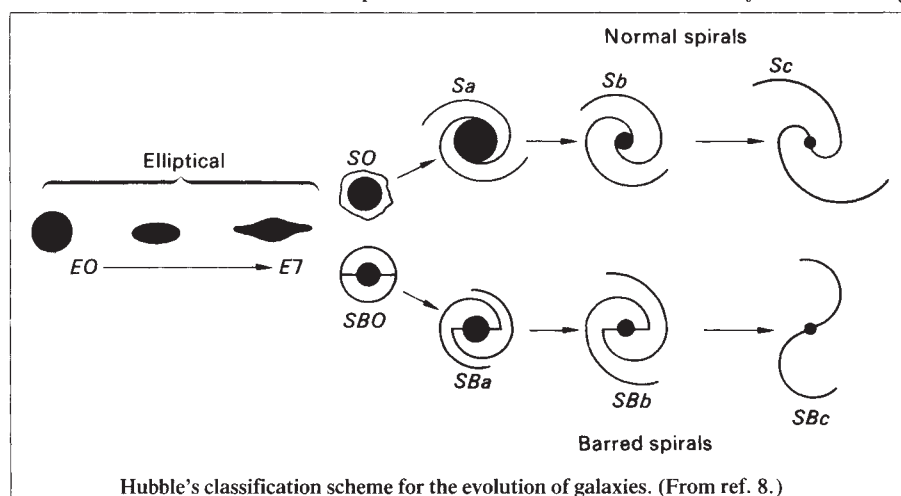
because spirals in general seem to have higher angular momentum than ellipticals.

Lake and Carlberg¹ have found a regulatory mechanism while investigating a dynamical 10,000-particle model. The model follows the fate of gas which collapses with an underlying distribution of dark matter. The latter, commonly assumed to exist in theories of the evolution of the Universe and inferred to exist from observations of galactic dynamics, interacts in the new simulations through its gravitational potential only. The models have essentially 90 per cent of their mass in this dark, unspecified

dispersion in the dark matter is higher (it is warm), the clumpy substructure does not form to such an extent and although some relaxation occurs to give a spheroidal component, densities do not get so great until much of the gas, keeping its high angular momentum, has managed to form into a disk, leading to a spiral galaxy.

One advantage of this new picture is that the redistribution of angular momentum during the formation of the spheroids helps to account for the wide differences in angular momentum content between spirals and ellipticals. The extent of this variation is hard to account for in conventional models⁴ in which tidal torques between forming galaxies generate the spin.

What is it that ultimately determines the critical velocity dispersion in the dark matter? Lake and Carlberg propose that the overall mass is responsible, with the rather more massive objects becoming



component — the usual inferred ratio. Star formation is simulated by a simple prescription of what happens when the gas 'particles' (or clouds) collide.

The final form of the distribution of stars and gas after collapse is found to depend critically on the magnitude of the random velocity dispersion initially present in the dark matter. The mechanism seems to work as follows. If the velocity dispersion is low (the matter is cold), the dark matter readily clumps together while the initial cosmological expansion changes to gravitational collapse. This clumping causes extensive violent relaxation — energy and angular momentum are redistributed among the particles (the gas clouds and dark matter) during the collapse. It is the transfer of angular momentum outwards in the cloud which is essential. The result is rapid star formation in the by-now dense and low-angular-momentum central regions, giving an elliptical galaxy. An embarrassment is that a high-angular-momentum gaseous halo is left around the outside, but the authors claim that this could easily be removed by other processes during the galaxy's normal evolution. When the initial velocity

elliptical galaxies. The link is not entirely obvious, and they admit that although total mass has often been invoked⁵ as a significant parameter controlling the Hubble sequence, it cannot be the whole story because the different Hubble types have overlapping mass ranges⁶. In determining the details of form, the ratio of spheroid mass to disk mass is obviously important, as the Hubble classification is to some extent based on the degree of spheroid dominance. But published observational determinations of bulge-to-disk ratios are probably much more unreliable than is often realized — photometric separation of disk and spheroid components is rarely unambiguous — and the mass of the spheroid may be as large as that of the disk in many spiral galaxies⁷. Once set by the initial dynamics, the bulge-to-disk

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ratio may well determine the resulting Hubble spiral class by influencing the formation of spiral structure and star formation rates in the disk. The exact conditions required for the presence of a bar remain rather obscure.

These interesting dynamical models have been investigated over only a very small number of possible parameter variations, and other variables still deserve investigation for their possible effect on

the final morphological type. This model implies that the era of galaxy formation occurred at a redshift of about three, beyond which only quasars have yet been observed. This work may show that it is futile to attempt to understand the formation process without taking account of the dynamical effects of the dark matter. □

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Cell growth factors

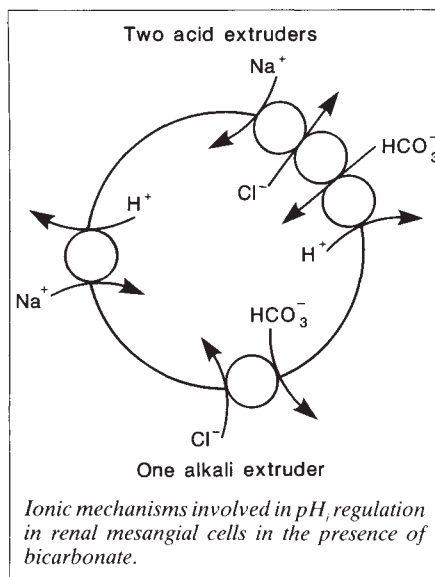
Bicarbonate and pH_i response

Roger C. Thomas

MUCH interest was aroused a few years ago by the first report¹ of continuous recording of the intracellular pH (pH_i) response to growth factors in cultured fibroblasts. That the fibroblasts came from human foreskins (*sic*) caused some twinges, but the main interest was the possibility that growth factors might stimulate growth, at least in part, by increasing pH_i . Moolenaar and his colleagues² showed that the pH_i increase was caused by stimulation of Na-H exchange, and later³ that protein kinase C activation was an intermediate step. But these experiments were done in the absence of bicarbonate, as pointed out at the time in a News and Views article by Boron⁴. On page 648 of this issue⁵, Boron and his colleagues now show that the growth factor arginine-vasopressin stimulates HCO_3^- transport more than it stimulates Na-H exchange. This result explains the earlier report⁵ that in solutions containing HCO_3^- and CO_2 , there is not only no pH_i increase, but the opposite happens — pH_i decreases.

Boron's group has recently shown⁶ that there are three distinct pH_i regulating mechanisms in the renal mesangial cells (related to smooth muscle) which they study (see figure). The first Na-H exchange appears to be the main mechanism for extruding acid from mammalian skeletal muscle⁷, and was until recently thought to be the main mechanism for acid extrusion in all mammalian cells. The second, sodium-dependent Cl-HCO₃ exchange, was first identified in squid axons (but without the sodium dependence⁸) and later found in snail neurons⁹, other invertebrate preparations¹⁰ and various vertebrate preparations¹¹⁻¹³. The third mechanism, as first described in cardiac muscle by Vaughan-Jones¹⁴, is one for alkali extrusion — to accelerate recovery from an alkaline load. This mechanism is simple Cl-HCO₃ exchange, as is also found in red blood cells where it is responsible for the Hamburger interchange or chloride shift. (There is in some preparations a fourth mechanism, Na-HCO₃ co-transport, but mercifully it does not concern us

here. Because Boron and colleagues in their latest studies did not record membrane potentials¹, they may have missed evidence for this electrogenic carrier.) The three mechanisms shown in the figure have not hitherto been identified all in one cell type, as far as I know. The only mechanism known to work without bicarbonate is Na-H exchange, inhibited specifically



by amiloride and related compounds.

Given that growth factors stimulate Na-H exchange it might seem unlikely that they would also stimulate the two bicarbonate-requiring mechanisms in renal mesangial cells. But Boron and his co-workers clearly show⁵ that all three mechanisms are stimulated. The dominant mechanism after stimulation is the alkali-extruding one. Thus the pH_i increase seen with growth factor in the absence of bicarbonate is under physiological conditions a pH_i decrease. Clearly, the Na-H exchanger is unlikely to be the transmembrane signal transducer originally proposed¹.

Why was the possible importance of bicarbonate ignored for so long? Its crucial role in pH homeostasis in the intact animal has long been recognized, if poorly

understood by most students. Inside cells, it is often the most common anion, and outside it is the second most common after chloride. But in practice, bicarbonate-buffered solutions are difficult to handle. For a stable pH the solution must be equilibrated with a gas mixture containing a known and fixed percentage of CO₂. Such gas mixtures are expensive and tend to vary from cylinder to cylinder. Carbon dioxide readily crosses plastic, especially silicone rubber tubing and leaves solution all too readily when pumped by a peristaltic pump, for example. In many optical systems it is simply much easier to avoid any gas at all.

Thus, when it was discovered that cultured mammalian cells could regulate their pH_i perfectly well with an amiloride-sensitive Na-H exchanger, it was widely assumed that bicarbonate would make no real difference. This assumption ignored the significance of bicarbonate in intracellular buffering^{10,15}, even without taking into account its requirement by pH_i regulators. Because the membrane is very permeable to CO₂, intracellular carbonic acid concentration is effectively clamped. Thus every intracellular bicarbonation can buffer four times better at any physiological pH than any ordinary buffer at its pK. For fast buffering, carbonic anhydrase is needed¹⁶. The lack of bicarbonate in the original experiment would thus have exaggerated any pH_i change due to stimulation of Na-H exchange.

There are two conclusions from this story. Intracellular pH is probably too fundamental a parameter to act as an intracellular messenger, even for an event as important as the initiation of growth except perhaps in acid-transporting epithelia¹⁷. As Boron has pointed out⁵, it is to be expected that growth factors will stimulate the housekeeping abilities of the cell such as pH_i regulation, in anticipation of the increased metabolic requirement of growth. The second conclusion is that he who works in bicarbonate-free media risks studying cellular and molecular pathology rather than physiology. □

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Palaeontology

New window on Proterozoic life

Andrew H. Knoll and Nicholas J. Butterfield

... if my theory be true, it is indisputable that before the lowest Silurian stratum was deposited, long periods elapsed, as long as, or probably far longer than, the whole interval from the Silurian to the present day; and that during these vast, and quite unknown, periods of time, the world swarmed with living creatures.

SURPRISINGLY, it took palaeontologists more than a century to prove the essential correctness of Darwin's speculation¹. When unambiguously Precambrian microfossils were described from a handful of localities in the 1960s, the minute filaments and unicells preserved in these rocks, notably the Gunflint and Bitter Springs formations, did more than establish the tremendous antiquity of life. They gave rise to a perception of Precambrian communities as vast expanses of cyanobacteria and other prokaryotes, and in younger, Proterozoic rocks, simple, possibly eukaryotic organisms. During the past 20 years, the discrepancy between this well-entrenched textbook notion and palaeontological reality has widened dramatically, as shown by the remarkable new fossil assemblage reported by Zang and Walter on page 642 of this issue². This discovery illustrates the considerable morphological complexity that was attained by late Proterozoic phytoplankton, and underscores the need for careful consideration of stratigraphy, palaeoenvironment and taphonomy (processes of fossilization) in the interpretation of early fossil assemblages.

The new assemblage, found in the 600–650-million-year-old shales of the Pertatataka formation in central Australia, includes about two dozen taxa of spinose, process-bearing or otherwise ornamented acritarchs, many of them extremely large relative to comparably ornamented fossils in younger rocks. (Acritarchs are organic-walled spheroidal microfossils of uncertain taxonomic status. Most are thought to be the cysts of prasinophyte, chromophyte or extinct groups of algae.)

The diversity of this assemblage is unprecedented, but there is a trail of discoveries that leads up to it. Even at the time of the Bitter Springs discovery, populations of morphologically simple

acritarchs were known from various Proterozoic shales of the Soviet Union³. By the early 1980s, there were enough data on these modestly sculptured acritarchs for a coarse but useful biostratigraphic zonation and to suggest a late Proterozoic diversification of eukaryotic phytoplankton, followed by a latest Proterozoic episode of extinction⁴. Compressions and cellularly preserved remains of seaweeds were also reported, as were vase-shaped

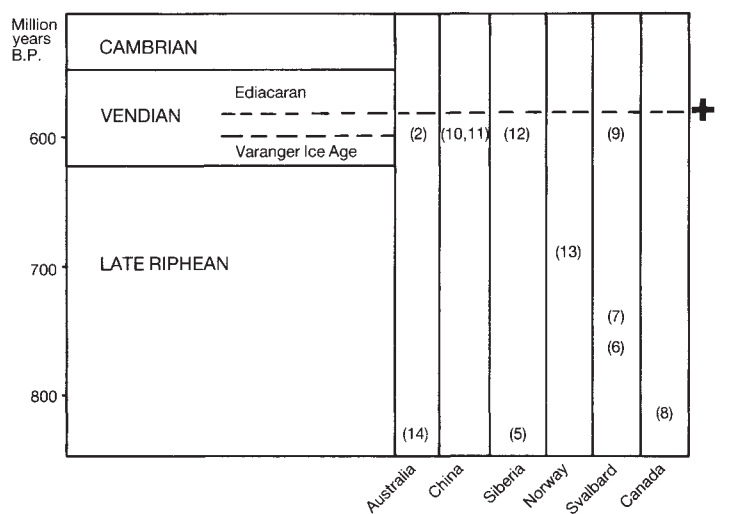
of Pertatataka acritarchs to other Proterozoic fossils is of more interest. Of the 10–15 acritarch genera we believe to be in the Pertatataka assemblage¹⁴, up to a half are unique to this formation, another third are genera previously reported from other Proterozoic formations, and only two or three are most closely related to characteristically Palaeozoic genera — and even here, as Zang and Walter stress, the Pertatataka fossils can be two to three orders of magnitude larger (by volume) than their younger counterparts. Indeed, the most remarkable aspect of these large complex acritarchs is that they do not appear to occur in Lower Palaeozoic rocks. Thus we believe that, together with other recent discoveries, the Pertatataka

fossils show that large, complex acritarchs were the characteristic fossils of the later Proterozoic era 900–600 million years ago.

Why is it that assemblages comparable to the Pertatataka were not found, or at least appreciated, earlier? One answer may be that many of these complex microfossils are preserved in subtidal cherts or very fine-grained mudstones, and possibly represent instances of special preservation. Zang and Walter, however, favour an environmental explanation for the unusual composition of their assemblage. They suggest that Pertatataka shales are distal turbidites, a deep-water rock formation little sampled by palaeontologists interested in Pro-

terozoic evolution. The fact that other comparable fossils occur in shallow-shelf deposits, however, suggests that other factors influenced their distribution.

Zang and Walter do not consider biogeographical and stratigraphical distinctions. It is possible, for example, that the morphologically simple acritarchs characteristic of latest Proterozoic shales from



Reported occurrences of large, ornamented acritarchs in Proterozoic sedimentary rocks (references cited in text). The wide geographic and temporal distribution of these fossils suggest the potential for an improved biostratigraphic zonation of late Proterozoic sequences. This distinctive microfossil grade appears not to be represented in Ediacaran or younger assemblages. Absolute dates for microfossil occurrences and stratigraphic boundaries are approximate.

protistan microfossils and large, complexly ornamented acritarchs of the type seen in the Pertatataka formation. The latter were first described from approximately 850-million-year-old shales from Siberia⁵, and subsequently from slightly younger shales and cherts from Svalbard^{6,7} and Arctic Canada⁸, as well as from latest Proterozoic rocks of western Svalbard⁹, China^{10,11} and Siberia¹² (see figure). *Papillomembrana*, a 400–500-μm ornamented vesicle from the approximately 700-million-year-old Biskopås conglomerate, Norway¹³, has conventionally been interpreted as a dasyclad alga, but it too probably belongs to this distinctive acritarch grade.

What do these acritarchs reveal about early evolution? The interpretation of fossils begins with systematic palaeontology, and taxonomic choices both reflect and influence the way we think about evolutionary pattern and process. In describing the Pertatataka fossils, Zang and Walter choose generic names that emphasize similarities to younger microfossils. But in our view, a comparison

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the Northern Hemisphere (mainly in the Soviet Union and Scandinavia) are not strict time equivalents of the Pertatataka biota. In China, large complex microfossils occur in formations immediately beneath strata bearing Ediacaran metazoans, but Ediacaran assemblages in the strict sense appear to consist only of simple spheroidal acritarchs and very small spiny microfossils (*Michrystidium* spp.)¹, much as in the Soviet Union.

Whatever the answer, Zang and Walter remind us of the untapped potential of Proterozoic fossils, among other things forcing a reconsideration of the timing of Proterozoic phytoplankton radiations and extinctions. The earlier hypothesis of an

end-Proterozoic extinction poorly constrained in time but probably coeval with Varangian glaciation must now be replaced by one of a major turnover just before the Ediacaran radiation. Perhaps the most important effect of the Pertatataka and other recently described fossil assemblages will be to displace the textbook caricature of Precambrian life with a view that emphasizes the biological diversity, ecological complexity and evolutionary dynamics of the pre-Ediacaran biota. □

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Marine geology

Sea-floor volcano erupts ideas

Joe Cann

Was there a huge volcanic eruption on the floor of the Pacific Ocean in 1965 that covered an area of 220 km² with 15 km³ of basalt lava? Such an eruption would have been comparable to the giant Lakagigur eruption in Iceland in 1783, which erupted 12 km³ of basalt, poisoned the island's grasslands, killed many of the animals and led to a severe famine¹. Yet the Pacific eruption was not observed at the sea surface, and the evidence can at present be only circumstantial. But even before its formal publication, the possibility has raised a kaleidoscope of new ideas in areas ranging from plate tectonics through hydrothermal activity to climate change. The case is made by Macdonald, Haymon and Shor in a paper shortly to appear in *Geology*² and draws evidence from various sources.

The primary evidence for the eruption comes from a survey of an area of the crest of the East Pacific Rise at 8–9° S. The East Pacific Rise is the mid-ocean ridge that separates the Pacific plate from the Nazca plate. At these latitudes about 0.2 m of new oceanic lithosphere is created on average each year by sea-floor spreading, the fastest rate known anywhere on the global mid-ocean-ridge system. The survey was made with the SeaMARC II towed side-scan sonar vehicle which acoustically measures ocean depths over a swath 10 km wide, and also constructs a side-scan sonar map, on which the intensity of backscattered sound is plotted on each side of the ship's track to show fault scarps (intensely reflecting strips), fissures (parallel shadows and intense reflections) and more complex shapes of volcanic seamounts (see the recent review article³ by Macdonald *et al.*).

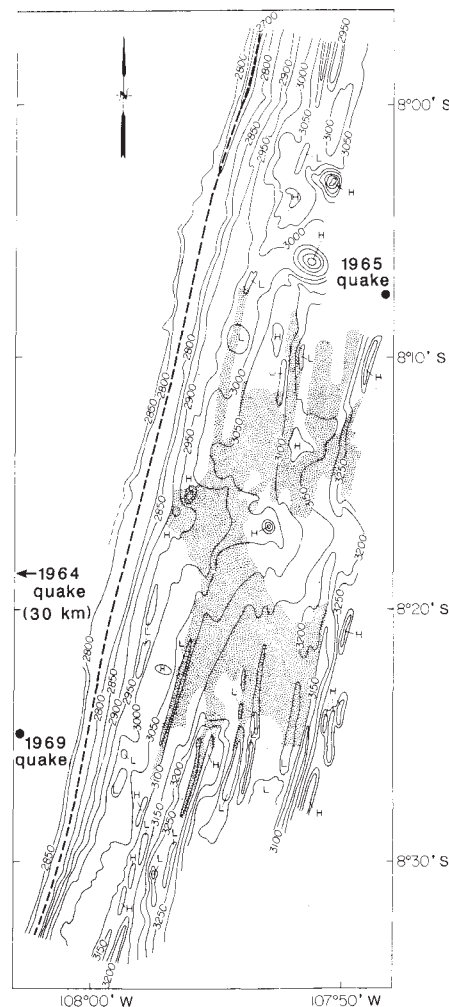
Between 8° 08' S and 8° 28' S, over a distance of 35 km, a few kilometres east of the spreading axis, marked here by a

1-km-wide shallow rift valley, appears an irregular area of high reflectivity (see figure). The bathymetry shows this area to be confined to topographic lows, to terminate against fault scarps and against a short chain of craters 3-km east of the rift valley. The plan shape is very similar to that of lava flows on the sea floor and on land, and there can be little doubt that this area is a lava flow emerging from the chain of craters. The thickness was calculated from where the flow abuts fault scarps, and the volume of 15 km³ follows from the surface area.

How young is the flow? High reflectivity has been correlated in other places with young, glassy lava flows still not covered by sediment. A date in the 1960s is suggested by three earthquakes recorded in 1964, 1965 and 1969 from this general area. It is very unusual to record earthquakes at fast spreading ridges except from the transform faults that offset them and the nearest transform fault is 100-km south of this area.

Suppose the lava flow was erupted in the mid-1960s. Could it be related to a giant plume of ³He-enriched sea water discovered in 1970–72 on the west side of the East Pacific Rise at 13° S by Lupton and Craig⁴? Magma from the Earth's mantle brings ³He up as new lithosphere is created by sea-floor spreading, and can be emitted into the ocean by volcanic gases, or stripped from lavas by hydrothermal solutions. In such young magma, the ³He/⁴He ratio is 100 times the atmospheric ratio, and inputs to the deep oceans can be traced thousands of kilometres from their source.

The plume was originally ascribed to very active hydrothermal venting at the ridge crest at 13° S carried west by deep ocean currents. Now, Lupton and Macdonald⁵ argue that the plume could



Map of the East Pacific Rise around 8° S, showing the extent of the lava flow (stippling) and sites of earthquakes in the 1960s. Contours, 50 m; broken line, spreading axis. (From ref. 2; courtesy of K.C. Macdonald.)

result from the volcanic eruption at 8° S, and that deep ocean currents would first have carried it south and then west at 13° S. This raises the first of the new ideas that have emerged from this work. The ³He plume has been used to estimate the steady-state hydrothermal flux into the oceans and to suggest that an especially active hydrothermal vent field lies at 13° S on the East Pacific Rise. If it has a volcanic source, that would alter the arguments drastically, and would require a re-examination of the whole question of hydrothermal flux of heat and elements. This idea is readily tested: if the plume was volcanic in origin, it should no longer be present, as eruptions of this kind must be very rare events.

The volume of magma erupted each year along the global mid-ocean-ridge system is about 3 km³ (assuming the thickness of ocean crust lavas is 1 km, as indicated by ocean drilling and ophiolite stratigraphy). The 8° S eruption would thus account for 5 years' world production of lava. Perhaps more relevant is the lava production at the 75-km-long spreading segment on which the eruption happened.

At the local spreading rate of 0.2 km yr^{-1} , the flow represents 1,000 years' supply of lava erupted at one place and time.

There are two ways in which new sea floor is created: by magmatic intrusion and extrusion to form new crust; and by stretching of pre-existing crust. When the latter happens on a large scale, as it does in the North Atlantic, the magnetic-anomaly pattern is degraded and outcrops of lower-oceanic crust are found at the sea floor. Such effects would be seen on the East Pacific Rise if eruptions occurred only once every 1,000 yr and each covered less than 10 per cent of the sea floor accessible to major eruptions. In 10,000 yr, about 2 km of new sea floor forms at these fast spreading rates, and most of this would have to be produced by crustal stretching if eruptions were episodic. In fact, the East Pacific Rise has a continuous lava carapace, outcrops are confined to fracture zones, and the magnetic anomaly pattern contains much fine detail. Probably no more than 10 per cent of lava can be erupted in major flows if both of these conditions are to be maintained, which would mean that the world-wide recurrent interval of major flows must be more than 50 yr, perhaps several hundred years. Indeed, the Lakagigur eruption in Iceland may have been the most recent eruption of this kind preceding the Pacific one.

Macdonald *et al.* discuss the effect of the eruption on the axial magma chamber that has been demonstrated to underlie the crest of the East Pacific Rise elsewhere. They estimate that the flow represents about 10 per cent of the volume of the magma chamber, but in doing so, I believe, they overestimate the size of the chamber: the flow may represent as much as half its volume. In either case, the eruption should have resulted in a significant deflation of the ridge crest at least in the local area of the eruptive craters. Yet the ridge-crest morphology is described as inflated and appears so on the map. If this is the case, then the eruption (which emerged apparently not from the crest of the ridge but from the base of the axial high 2–3 km from the spreading axis) must have resulted from some extra inflation, perhaps from the sudden injection of a large volume of new magma into an already inflated magma chamber.

This presents another fascinating aspect of the problem. Recently McKenzie and colleagues⁶ succeeded in convincing many volcanologists that magma must dribble out of the mantle as soon as it forms, and that large bodies of magma cannot form within the viscous asthenosphere which underlies the mantle. If here we have a spectacular counter-example, suggesting a sudden input of several cubic kilometres at once into a magma chamber at one place, that would have a profound effect on this line of thought.

By coincidence other apparently young

and massive sea-floor lava flows have been discovered recently⁷. But these are not at spreading plate boundaries. Instead they cover large areas of the deep ocean floor around the Hawaiian islands. The flows were discovered during a systematic survey of the US exclusive economic zone with the British side-scan sonar GLORIA. In one place the flows cover $150,000 \text{ km}^2$, and fresh glassy basalt has been recovered, showing that the flows are young. It is not clear whether these flows are related to the island volcanoes (they are found in deep oceans 1–300-km offshore) or to the magmatism of plate boundaries (they are in the centre of the Pacific plate). Whatever the case, they clearly represent another submarine volcanic enigma.

The discovery of these apparently young, large lava flows has led Shaw and Moore⁸ to speculate that large sea-floor eruptions might be a trigger for the El Niño climatic fluctuations of the equatorial Pacific. They suggest that the amount of heat released from a young lava flow of this size would be enough to produce sufficient warming of the sea surface layers to generate climatic fluctuations. This, I think, is the easiest of the ideas spawned by the East Pacific eruption to test. Not only would the frequency of such eruptions be too low to generate the observed frequency of El Niño, but Shaw and Moore entirely neglect the problem of getting the heat to the surface. For a plume of hot water to penetrate the stratification of the deep ocean, and then the thermocline, in the well-stratified equatorial Pacific, would apparently require a buoyancy flux of far greater magnitude than could be provided by even a large eruption. Surely that trail must be a dead end.

But if a single observation has generated so many interesting sparks even before its formal publication, there must be fair mileage in it yet. The next step must be to go and examine the lava flow, to collect samples for analysis, to determine its helium-isotope content and to determine its age (which will not be easy). The helium plume must be revisited as a matter of urgency to test that side of the story. Then, of course, it might be a good idea to have another look at the more accessible Lakagigur flow. How does that fit into the scheme of global and Icelandic magma plumbing? How is that related to processes in the mantle and to the history of Icelandic volcanism? We'll see. □

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Daedalus

Time is money

QUEUEING, especially in long institutional queues, implies that while the time of the institution is valuable, that of the queuer is worthless. This outlook probably reaches its high point in the Soviet Union, where you can queue indefinitely for a permit to join another queue; but high marks must also go to the packed waiting rooms and overbooked appointments systems of Britain's much-loved National Health Service.

Daedalus now presents a practical solution. The DREADCO Op-time-miser[®] is a sort of combined register, computer and vending machine. Installed in (say) a hospital waiting room, it accepts details of each patient to transmit ahead to the doctors. It also notes his time of arrival, and requests him to state the value of his time in pounds per hour. It already has this information for those already present: so it can calculate a 'break-even waiting time' for the group as a whole. Those who value their time highly are assigned a shorter waiting time than this, and pay for the time they save; those who value it modestly wait longer, and are paid their stated rate for the added inconvenience. The 'break-even time' is calculated so that the money paid in by the short-stayers is just sufficient to recompense the long-stayers. The group transaction always breaks even.

Thus the usual dreary waiting for broken appointments is eliminated. Patients just turn up out of the blue and immediately receive an estimate of their waiting time and its likely cost or reimbursement. This time may change as later arrivals modify the optimization; but each queuer knows how much he will be paid for added inconvenience, or charged for speedier service. The rich, as ever, can buy their way to the head of the queue; but the poor are not aggrieved, as they get the money. Nobody will put an absurdly high value on his time; for that increases the chance that he will have to pay out at that rate. But a deposit may have to be charged, to deter healthy layabouts who set a very low value on their time so as to sit endlessly in the warm waiting room at a slowly growing profit.

The Op-time-miser will revolutionize public life. In hospitals, passport offices and unemployment bureaux, wherever an inflexible bureaucracy confronts a resentful public, it will rationalize and smooth their dealings. Daedalus also plans to sell its software to all those booking, repair and installation services that have to juggle unpredictable customer requests into a queue. It will compensate for the sort of maddening delays and broken promises that gave rise to this announcement in the 'Births' column of a British national newspaper: "To Mr and Mrs Smith. Gratefully, after seven years. A telephone". David Jones

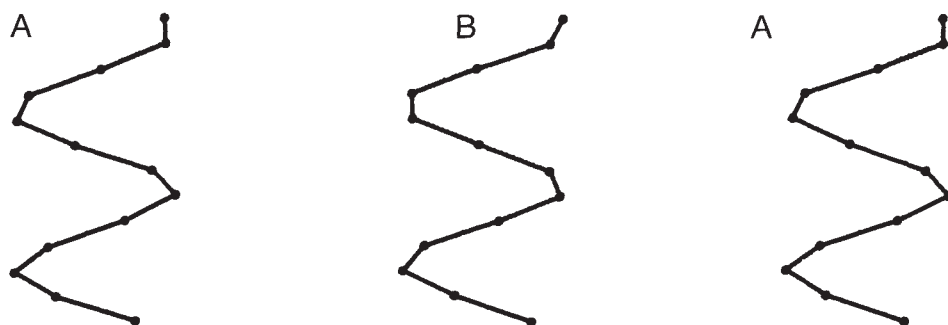
Ambiguities in stereopsis

SIR—Stereo-pairs, usually of molecules, are regularly printed in many journals and add a useful third dimension to the traditionally two-dimensional printed page. I

members exchanged in position. For a given viewing method, the two pairs yield three-dimensional images with reversed depth and handedness. Surprisingly,

retinal images of any pair shifts the retinal images of the entire row out of register by one member on each retina.

Stereo-pairs as illustrations of three-dimensional objects are now easy to produce with microcomputers and three-dimensional drawing programs. Authors



A stereo-triplet of a helical structure. Stereopsis of any two adjacent members yields two three-dimensional images with reversed depth and handedness. With crossed visual axes, the image on the right is right-handed, like a conventional screw thread. With uncrossed axes, it is left-handed.

wonder, however, how many readers actually see stereo-pairs in three dimensions with correct orientation. An informal poll of my associates reveals that almost no one keeps a stereoscope handy; only about half knew that stereo-pairs could be viewed in three dimensions with the naked eye (direct stereopsis); about a quarter can use direct stereopsis; and only one person knows that a stereo-pair is ambiguous; it does not distinguish between right-handed and left-handed three-dimensional structures.

The ambiguity arises because a stereo-pair can be viewed by two methods¹. Conventional stereoscopes use the uncrossed visual axes method — lenses, prisms or mirrors cause the right and left eyes to view the right and left members of the pair, respectively. But stereoscopes that use the crossed visual axes method can also be constructed. In these, the right eye views the left member of the pair, and *vice versa*. The depth dimension reverses with a change in viewing method, and the same stereo-pair that gives a right-handed three-dimensional image with one method gives a left-handed image with the other. In effect, the right and left members of the pair are exchanged in position.

Either method can also be used in direct stereopsis^{2,3}, but the crossed axes method seems easier to learn^{2,4}. Therefore, most people who use direct stereopsis will see a reversed image of a printed stereo-pair because the images are usually arranged for conventional stereoscopes, which use the uncrossed visual axes method.

A stereo-triplet (suggested by Bruce Nicklas) consisting of equally spaced members A–B–A, illustrates the reversal (see figure). If A on the right is covered, A and B on the left form a stereo-pair. If A on the left is covered, B and A on the right form the same stereo-pair but with the

viewing either pair with all members of the triplet uncovered yields two three-dimensional images side by side, reversed in depth and handedness. In general, a row of n members yields $n-1$ three-dimensional images with a two-dimensional image at each end of the row. This pattern occurs because fusion of the

should make such illustrations unambiguous by stating whether they are arranged for viewing with crossed or uncrossed visual axes.

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Earthquake records

SIR—Roger Bilham¹ has made a powerful case for a global approach to the long-term forecasting of earthquakes and, more specifically, for the use of the techniques of space geodesy to detect premonitory ground deformation. Although strain is a fundamental measure of the energy released by earthquakes, it may not always be recognizable at the surface using geodetic techniques. Therefore, we need also to understand the historic and prehistoric record of earthquakes in much greater detail than we do at present.

Long and well-dated earthquake chronologies² bring other benefits: they may reveal seismic zones that are temporarily inactive³ and they are bound to throw light on the mechanisms responsible for the earthquakes⁴.

In any case, the geodetic approach requires information on the strain release of a complete earthquake cycle at the site in question. In other words, it will not pay off until the next earthquake. As Bilham observes, that may be decades or centuries in the future: one of the primary tasks of palaeoseismology is to seek out any periodicity (or its lack) in the local occurrence of earthquakes of different sizes.

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Island occupation

SIR—In his recent News and Views article¹ on the initial human occupation of the islands of the south-west Pacific, Diamond asked the rhetorical question of whether Pleistocene colonists had managed to reach “only the largest visible targets, leaving Bismarck outliers to be discovered tens of thousands of years later by Lapita explorers?” The answer, as far as a south-eastward thrust down the islands of the Solomon chain is concerned, is no.

Wickler and Spriggs last year investigated² Kilu cave on Buka Island, just north of Bougainville Island in the northern Solomons (see map in ref. 1), and found late Pleistocene occupation deposits spanning the period from 20 to 28 kyr ago. These levels contain evidence for the exploitation of marine gastropods, fish and, judging from residue analysis of starch grains on some stone flakes, vegetable tubers. Being an oceanic island, the land fauna is depauperate but included some endemic murids which became extinct, probably because of human impact. This pattern of exploitation is similar to that shown³ in the Pleistocene occupation levels at the coastal cave of Matenkupkum, New Ireland, 33 kyr ago.

Buka is 175 km away from New Ireland and cannot be seen from there. Between the two islands are the small Feni and Nissan groups, but a journey from New Ireland to Buka via the Feni group would still involve a sea crossing of at least 145 km; and a crossing using both Feni and Nissan groups would be not less than 70 km. Nissan is a coral atoll, and it is not

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The face of justice?

John C. Marshall

Identifying Ivan: A Case Study in Legal Psychology. By Willem A. Wagenaar.
Harvester-Wheatsheaf/Harvard University Press: 1988/1989. Pp. 187. £13.95, \$20.

ALL trials are, or should be, 'show trials'. In accordance with the axiom "Justice must not only be done, but must be *seen* to be done", they should show as unambiguously as possible whether the accused is innocent or guilty as charged. Nazi and Stalinist show trials were obnoxious not because they were spectacles, but because those found guilty were so manifestly innocent.

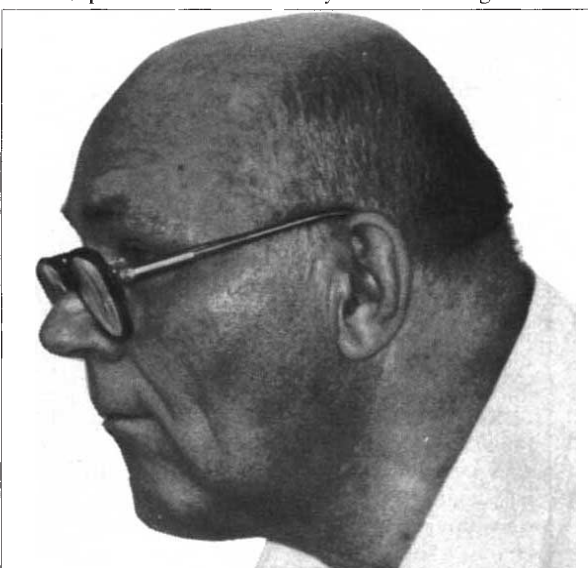
Last April, a Jerusalem court sentenced John Demjanjuk to death after a trial explicitly stage-managed to remind Israel's youth of the Jewish past in Europe; his appeal is pending. The crime is not at issue. A Ukrainian guard at Treblinka death camp was, in part, responsible for the murder of 850,000 Jews in the gas chambers. The court decided that this man, known to the prisoners as Ivan the Terrible, was John Demjanjuk, a factory worker from Cleveland, Ohio, who had been extradited from the United States to stand trial in Israel. Demjanjuk's defence was simple in principle, although just how complex in fact is revealed by Willem Wagenaar's monograph *Identifying Ivan: A Case Study in Legal Psychology*. The defence was mistaken identity — that Demjanjuk was not 'Ivan'.

Willem Wagenaar is a distinguished Dutch scientist who holds the chair of Experimental Psychology at the University of Leiden. His areas of professional competence include the study of memory and perception. He is an acknowledged expert on identification procedures and eyewitness testimony, and it is in this capacity that he agreed to act in the defence of John Demjanjuk. Other equally qualified psychologists had declined to so testify, fearful apparently of the reactions of friends and colleagues. Wagenaar's decision (and the whole tone of his book) is evidence of his intellectual and moral strength; both his character and the technical nature of his testimony have subsequently been vilified and distorted by many who ought to know better.

Quite apart from the specific discussion of Demjanjuk's case, the book is a vivid tutorial on the reliability (or otherwise) of facial identification and the rules for the conduct and interpretation of identity tests. These are areas to which experimental psychologists have made substantial contributions and have succeeded in

elucidating a wide range of perceptual, memorial, cognitive and social factors that affect the validity of identification judgments; studies of this topic deliver highly pertinent information to counter those who argue that empirical psychology is conceptually vacuous and socially useless.

Wagenaar summarizes the relevant experimental evidence superbly and provides an acute analysis of its strengths



Ivan the Terrible? Demjanjuk at a preliminary hearing in 1986.

and weaknesses; he falls into neither the trap of assuming that the expert is always right, nor that of dismissing scientific work because the knowledge so obtained is itself limited and fallible. Furthermore, he convincingly relates such laboratory studies to well-documented cases where flaws in line-up and show-up procedures are known (in retrospect) to have resulted in a miscarriage of justice. A number of Wagenaar's conclusions were prefigured in the document prepared by a British advisory committee, chaired by Lord Devlin (1976). Wagenaar pays due tribute to the Devlin Report, although the pace of research has now materially added to the body of information available. His monograph should accordingly be obligatory reading for all judges and lawyers — or better, for all citizens.

The central section of *Identifying Ivan* is a description and justification of 50 procedural rules for the fair conduct of identity parades. They include such seemingly reasonable requirements as (Rule 17): "Witnesses should, before participation in an identification test, be requested to

provide a verbal description of the criminal, as they remember him. These descriptions should be included in the reports of the subsequent identity test". And (Rule 21): "The instruction to the witnesses should stress that the wanted person is possibly not in the parade or photospread, and that therefore a positive response should be made only when the witness is certain of recognizing that person".

It is Wagenaar's contention that of the 46 rules applicable to Demjanjuk's case, 28 were directly broken and another nine violated by implication from earlier flaws. Similar procedural errors were made, Wagenaar claims, at the Cleveland trial in 1981 when Demjanjuk's US citizenship was revoked. It is true, as Wagenaar readily concedes, that not all of these rules

are accepted as an international standard. Nonetheless, the least that can be said on the basis of his quietly rational documentation is that justice was not *seen* to be done. Such violations could not, of course, occur in Britain. But at a time when the British government is attempting (with some success) to turn the state into an appendage of MI5, we should perhaps be aware of the problem.

Professor Wagenaar does not pretend to the wisdom of Solomon, and he is far too intelligent a man to dispute the verdict of the Jerusalem court. That decision was based upon more evidence than is available to an expert witness for the defence. As Wagenaar states: "The process of translating prior odds into posterior odds is influenced by the experts' likelihoods, but not determined by them". For the reader who wants to know the Truth, Wagenaar "can only refer to the court's verdict". It is, he writes, "the best answer we have".

I do, however, take away from his book one firm conclusion. We can all agree that no legal process will be perfect (at least until the Messiah comes); but there is, to my mind, a strong case to be made that *all* expert testimony should be presented not by 'experts' for the prosecution or the defence, but rather by 'friends of the court'. Until that day, Wagenaar might like to recall an old Yiddish story: The rabbi's wife was convinced that her servant girl was stealing from her. She told the rabbi that she intended to take the girl to court. When the rabbi said that he too would go, the rebbetzin informed him that she knew the law as well as he did and could successfully prosecute on her own. "Ah, yes", said the rabbi, "but who save me will dare to testify in your servant's defence?". □

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Fields in chaos

Ian Percival

Nonlinear Physics: From the Pendulum to Turbulence and Chaos. By R.Z. Sagdeev, D.A. Usikov and G.M. Zaslavsky. Harwood: 1988. Pp.675. Hbk \$98, £55; pbk \$39, £21. Six accompanying diskettes \$77, £43.

THE main authors of this book, Sagdeev and Zaslavsky, have made notable contributions to nonlinear physics. A few years ago Zaslavsky moved from Krasnoyarsk to work with Sagdeev at the Space Institute in Moscow, and the book is one result of their collaboration.

It is an ambitious venture, written for the physicist who wants to use nonlinear dynamics, and includes many analytic and numerical methods and results that have been developed in the past few decades. Its particular strength is the recognition of the important role of chaotic dynamics in many fields, particularly plasma physics. The emphasis is strongly on hamiltonian systems and on applications.

The authors begin with the more traditional methods of hamiltonian dynamics, but from a modern point of view. Then follows a section of special methods; these are largely developments of Poincaré's ideas as tools for applications, but include the KAM theorem. The next hundred pages deal with various aspects of chaos, most of it hamiltonian. There is a short chapter reviewing the principles — which derive largely from the work of mathematicians — and then a long one giving a detailed description of some of the complicated and subtle properties of hamiltonian chaos; most of these have been discovered

by numerical experiment using computers, but backed by theory. The next chapter, on kinetic theory, concentrates on those aspects of transport which are of importance in plasma physics.

Part 2, on nonlinear waves, starts with stationary waves, including normal and collision-free shocks, ion-acoustic and magnetosonic waves; the connection made with the theory of particle motion is helpful here. Exactly integrable wave equations are introduced in the context of their applications, and are then treated in more detail later. There follows a description of the hamiltonian formulation, including resonant interaction, resonances and interaction of phonons, and two long chapters on chaos in wave fields and strong turbulence. Strong turbulence is introduced through the Lorenz model, and the distinction between temporal and space-time chaos.

Part 3 gives examples, and is really a collection of bits and pieces that might not have fitted naturally into earlier sections. The first and best is on the motion of particles in wave fields, to which the authors have made important recent contributions and which are illustrated with strikingly beautiful computer-produced colour plates at the end of the book. The second is on billiards, but is too brief to do justice to the subject. The connection with the ray theory of wave optics is considered, but there is no mention of the work of Keller, Maslov, Gutzwiller, Berry and many others on the connection between rays and waves in general, and billiards and cavities in particular.

A later section deals with nonlinear chains, especially with the mathematical equivalence of the equilibrium of incommensurate structures and the dynamics of certain maps. Here, however, there is no

description of the Frenkel-Kontorova model, or of Aubry's classic work on its correspondence with the standard map. There are also chapters on applications to nonlinear optics, and to the perturbed Kepler problem (with applications to astronomy and to the excitation of Rydberg atoms). Each provides enough to whet the appetite, but falls far short of being a modern review of the field.

The fourth and final part of the book, by Usikov, is the most unusual. It provides a detailed guide to the programming methods necessary to explore some of the dynamical systems with the aid of an IBM-compatible microcomputer. The detailed listing is not provided, though disks can be obtained at an extra cost.

Part of the problem in writing a book of this kind is that the detailed theory is presented analytically, with some help from geometry, whereas many of the advances are made with the aid of computer experiments, which cannot be put directly into print. So the normal method of presentation is misleading. The final part of the book tries to redress this situation. The 'colour supplement' of orbit simulations is valuable, and will encourage readers to experiment for themselves. But it is not so clear that the computer programs will be so useful, and there is a strong risk that they are becoming outdated — nowadays people use mice!

It is said that Enrico Fermi's use of English was often wrong, but that it was best not to correct it because the errors were minor and had such charm that it was a pity to remove them. Much the same can be said of this book — for example "multiple period birth bifurcations" (p.199) looks more like a term from gynaecology than nonlinear dynamics; but we know what the authors mean. More serious is the use of the words "chaos", "stochasticity" and "turbulence" without the distinctions of modern usage, and the use of "soliton" for interacting solitary waves.

My impression is that the first three parts of the book were written by two busy and productive scientists, who naturally concentrated on the areas to which they have made research contributions. They did not, it seems, have the time to bring all the chapters up to date, nor to provide the polish, revision, indexing, references and cross-references which would have made this a much better volume. For example, although the references provide a guide to some important Soviet literature, they are by no means comprehensive.

Nonetheless this is a valuable contribution to the literature on nonlinear physics. And unlike Zaslavsky's earlier book *Chaos in Dynamical Systems*, which was terribly overpriced, it is very good value for money, especially in paperback. □

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Living totem pole — "Young Condor on Post, Steamboat in Background, Jennings Lodge, Oregon, 1906", a high-quality photograph on a 5 × 7-inch glass plate by W. L. Finley and H. Bohlman. The picture is taken from a chapter on printing old negatives in Alfred A. Blaker's *Handbook for Scientific Photography*, 2nd edn, just published by Focal Press (an imprint of Butterworth), price \$45, £39.95.

Issues of organic origins

James P. Ferris

The Emergence of Life: Darwinian Evolution from the Inside. By Sidney Fox. *Basic Books: 1988. Pp. 208. \$17.95.*

SIDNEY FOX, a pioneer experimentalist on the origins of life, has vigorously promoted research into the subject and his particular approach to it since the 1950s. His work has prompted debate and controversy, and has sparked interest in the field. In *The Emergence of Life*, he summarizes his findings and conclusions for those with a basic understanding of high-school biology. Readers, however, should take to heart the warning to be found in the preface: "I feel comfortable with a frankly acknowledged bias derived from experiments and experience". It appears that bias has indeed played a strong role in the development of his ideas. In this review I will examine in detail two of the basic precepts of Fox's theory which I believe are built on distinctly wobbly experimental foundations.

Polymers

The first is that ordered polymers are formed by the thermal polymerization of amino acids. Fox's view that protein was the basis for the first life stems from his observation that polymers are formed by the dry-phase heating of amino-acid mixtures which contain an excess of either glutamic acid, aspartic acid or lysine up to 180°C. These polymers are not linked simply by peptide bonds; rather, many of the bonds (33–100 per cent in the case of polymers formed with excess aspartic acid) arise from chemical interaction with the carboxyl or amino groups that are not involved in normal peptide bond formation. Fox clearly feels that they are protein-like and justifies this in part because *Chemical Abstracts* indexes them as "thermal proteins". But indexing is one thing, experimental evidence is another.

Is the thermal polymerization of amino acids on the early Earth a plausible idea? It is not beyond the realm of possibility, but other questions remain. For example, how did the excess of acidic or basic amino acids accumulate? Aspartic and glutamic acids are among the more abundant amino acids formed in experiments simulating the primitive Earth. But glycine and alanine are usually formed in much larger amounts, while lysine is formed in trace amounts only. So it is difficult to understand how the basic thermal polymers came into being. Other organic substances may have inhibited the polymerization process because it is highly unlikely that one would find an area on the primitive

Earth where only amino acids concentrated. For example, the effect of carboxylic acids and amines on the thermal polymerization of amino acids merits investigation, because they may have accumulated together with acidic or basic amino acids.

It may be possible to overlook problems with the polymerization thesis if Fox's proposal that there are internal limitations on the structures of these thermal polymers is correct. The proposed internal ordering is the basis for an even more controversial assertion — that molecular memory of the type provided by DNA in contemporary life was not essential in the earliest life.

One experiment cited by Fox involved the copolymerization of a 1–2–1 mixture of glutamic-acid–glycine–tyrosine where only two tyrosine-containing tripeptides were isolated from the reaction mixture (higher molecular weight oligomers were also formed). These tripeptides have the composition pyroglu–gly–tyr and pyroglu–tyr–gly. Fox claims that this result is evidence of internal ordering, because there are 19 different ways in which three amino acids containing one or more tyrosines could be linked, yet only two were observed. This reasoning does not stand up to more detailed scrutiny. Polymers form in the presence of glutamic acid because it cyclizes rapidly to pyroglutamic acid first, and then amide bonds are formed with the carboxylic acid or imide groups of the pyroglutamic acid. Consequently, only a glutamic acid residue will be on the amino terminus of the chain. In reality, this is a system where the location of glutamic acid is fixed so only three tyrosine-containing dipeptides will be formed. In the experiment, the molar amount of glycine was initially twice that of tyrosine, and because tyrosine decomposes more readily than glycine under thermal polymerization conditions it is hardly surprising that the trimer containing two tyrosines was not detected among the reaction products. Thus the observation of only two tripeptides is consistent with the known chemistry of the reactants used and cannot be generalized to more complicated mixtures of amino acids.

The complexity of the reaction product mixture formed from 18 amino acids was revealed when it was subjected to DEAE-cellulose chromatography after initial purification by dialysis (Dose, K. & Rauchfuss, H. in *Molecular Evolution, Prebiological and Biological* (eds Rohlffing, D.L. & Oparin, A.I.) 199–217; Plenum, 1972). Preparations which appeared to be homogeneous in gel electrophoresis could be split into more than seven fractions. Even then it was not established that any of the fractions were homogeneous. No self-ordering was observed in this fractionated material, and indeed Fox never reports unambiguous data for self-

ordering in thermal polymers. For example, critical experiments to define self-ordering, such as nearest neighbour analysis or sequence specific enzymatic hydrolysis, are not presented.

Protocells

The second of Fox's precepts with which I disagree is that protocells are formed from thermal polymers. When thermal polymers are dissolved in a small amount of boiling water and the mixture is allowed to cool, the polymers (which are insoluble in cold water) precipitate out as spheres 0.5–7 µm in diameter. Fox refers to these particles as microspheres, protocells or primordial cells. Microscopic examination of the particles indicates that there is a dark boundary layer at their surface which looks like a membrane. The apparent presence of boundary material suggested to Fox that if these spheres formed on the primitive Earth, they may have encapsulated other organic material and this system eventually evolved into the first cell. Unfortunately, most of the microsphere material dissolves when the solution in which they form is diluted with water or buffer, while the dark material that looks like a membrane dissolves partially. Fox tested the permeability of this dark material to carbohydrates by forming the microspheres in the presence of solutions of glucose, fructose, starch or glycogen. The microspheres were washed with water and after four washes there was still glycogen or starch remaining in the fraction with the dark material, but fructose or glucose was completely removed. But Fox reports only a few experiments, so it is impossible to determine how much of the original encapsulated carbohydrate remained after washing the microspheres (Fox, S.W. *et al.* in *Physical Principles of Biological Membranes* (eds Snell, F. *et al.*) 417–432; Gordon & Breach, 1970).

Finally, Fox and his colleagues noted in the same article, "How much of this [bound carbohydrate] is attributable to the boundary is not known". Thus, whether the carbohydrate is bound to the dark material or whether it is really retained inside a cavity formed by the boundary material was not established. There are no reports of the encapsulation of authentic protein or nucleic acid within the microspheres. Microparticles (no boundary layer) are formed between lysine thermal polymers and nucleic acids as a result of the charge interaction between the protonated lysine and the phosphate anions of the nucleic acids. One would expect that nucleic acids would be retained inside the microspheres formed from acid thermal polymers if there was any sort of a membrane present, but such studies were not reported. Fox has little experimental support for the assertion that there is a lipid-like membrane at the surface of these microspheres

— it seems that no structural analyses of the presumed boundary material were carried out. The failure to retain thermal polymers, and the absence of data on the retention of protein and nucleic acids, leads me to the opinion that microspheres formed from thermal polymers could not have served to encapsulate the first life on the primitive Earth.

Because these fundamental proposals have such poor experimental support, I am forced to conclude that Fox's theory has little validity. Consequently, the other claims to be found in the book — on the significance of the budding of microspheres, their implied sexuality, the 'membrane' potentials and so on — are meaningless in the context of the origins of life. This is not to say that polypeptides did not have a role in the first life, or that there might have been some sequences that formed more readily than others, but it does not seem likely that proteins with

specified sequences formed without some sort of a catalyst (code?) to direct their formation. Investigation of syntheses using conditions that are more selective than dry, thermal polymerization may shed some light on prebiotic peptide formation.

On the positive side, the book provides a lively account of Fox's ideas and of how they developed. Fox is a strong proponent of the view that the most useful insights for the understanding of the origin of life come from knowledge of the products of potentially prebiotic precursors and not from the examination of contemporary and ancient biochemical systems; that is a view that is gaining wider acceptance today. But most of the conclusions that he has drawn far exceed what is justified by the data. □

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Set and match

Ian Stewart

Fractals Everywhere. By Michael Barnsley. *Academic: 1988. Pp. 396. £27, \$39.95.*

Not too long ago there was only one book on fractals, Benoit Mandelbrot's *The Fractal Geometry of Nature*. Now there are several, but here we have the first genuine textbook on fractals as fractals (the admirable *Geometry of Fractal Sets*, by Ken Falconer, is too much a textbook on geometric measure theory to count here). Michael Barnsley has taught the material at the Georgia Institute of Technology, where his course is open to all students who have completed two years of calculus; the resulting volume could be used by final-year undergraduates or at postgraduate level.

Barnsley concentrates on the mathematics that underlies the theory of fractals: the only application discussed is to data-compression in computer graphics. He takes pains to convey the ideas behind the theorems, using diagrams as well as formal definitions to put across abstract concepts such as metric space, affine transformation and Hausdorff dimension.

The mathematical core of the book is the notion of a self-similar set, which is fundamental to the fractal point of view. A set is self-similar if there exists some finite system of transformations — or iterated function system — such that the images of the set under these transformations, when combined, yield the original set. In other words, the set can be split into a finite number of pieces, each of which has the same structure as the whole, but on a smaller scale. Every iterated function system possesses a unique invariant set —

that is, a set which is self-similar under that system. This invariant set is in general a fractal, except for unusual choices of the function system.

One of the central results concerns the 'chaos game'. If an initial point is chosen, and transformations from the function system are applied to it at random to create new points, then with probability one the set of points so formed becomes closer and closer to the unique invariant set for the system. This forms the basis of a computer algorithm for drawing the invariant set. Barnsley deals in depth with the mathematical and dynamical features of the chaos game (including the fractal dimension of the invariant set), alongside its application to computer graphics. The ideas are also extended to a probabilistic version, in which the black-and-white decision 'point' or 'no point' is replaced by shading on a scale of grey. The mathematics behind the famous Mandelbrot set, and its associated Julia sets, is discussed in some detail.

This is a fetching book, with beautiful computer graphics (some in colour), as well as a host of more schematic diagrams, the aim of which is to clarify the abstract mathematics. The fractal ferns, in particular, are quite remarkable, and relate directly to the central ideas. The material is well chosen and hangs together coherently, and the writing conveys a proper sense of excitement. It is true that some of the proofs are tough going, and demand a certain mathematical maturity; but the book can be understood even if the occasional proof is set to one side for future investigation. If you want a genuine understanding of fractals as mathematics, and not just as attractive images, this is an excellent place from which to start. □

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Innervative ideas

Alun M. Davies

Body and Brain: A Trophic Theory of Neural Connections. By Dale Purves. *Harvard University Press: 1988. Pp. 321. \$35, £27.95.*

It is common knowledge that the brain controls the body. That the body has an organizing influence on the nervous system is not generally appreciated, yet maturation and growth in all but the simplest of organisms is accompanied by the continuous adaptation of neural connections to the body's changing needs. Purves takes this adaptation as the starting point for the exposition of a trophic theory of neural connectivity. The theory not only provides an important unifying principle in neural development, but has implications for several diverse areas of biological science.

In the context of the nervous system, the term trophic refers to the long-term interdependence between neurons and the cells they innervate. In development, these trophic interactions regulate the size of neuronal populations and have a part in modifying the extent and complexity of neural connections throughout life. Purves presents a comprehensive overview of the foundations of trophic theory from its origins in experimental embryology to our current understanding of its molecular basis.

Alongside the central theme of trophic interactions in development and growth, there is discussion, from an evolutionary standpoint, of the significance of the adaptability of the nervous system to changes in body size and form. The link between neural activity and the modification of connections is examined in terms of trophic theory, an issue which has a bearing on the molecular basis of learning. Finally, Purves evaluates the currently fashionable regressive theories of neural connectivity that are based on a direct analogy between neural development and natural selection. His arguments clearly expose the inadequacies of the naive view that the nervous system develops according to some abstract darwinian principle.

This is a scholarly, thoughtful and well-written book. It will be of value not only to neurobiologists but to anyone interested in basic biological issues. □

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New in paperback

Cerebral Dominance: the Biological Foundations edited by Norman Geschwind and Albert M. Galaburda. Publisher is Harvard University Press, price is £10.50, \$12.95. For review see *Nature* 314, 475 (1985).

Radiocarbon dating of the Shroud of Turin

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Very small samples from the Shroud of Turin have been dated by accelerator mass spectrometry in laboratories at Arizona, Oxford and Zurich. As controls, three samples whose ages had been determined independently were also dated. The results provide conclusive evidence that the linen of the Shroud of Turin is mediaeval.

THE Shroud of Turin, which many people believe was used to wrap Christ's body, bears detailed front and back images of a man who appears to have suffered whipping and crucifixion. It was first displayed at Lirey in France in the 1350s and subsequently passed into the hands of the Dukes of Savoy. After many journeys the shroud was finally brought to Turin in 1578 where, in 1694, it was placed in the Royal Chapel of Turin Cathedral in a specially designed shrine.

Photography of the shroud by Secondo Pia in 1898 indicated that the image resembled a photographic 'negative' and represents the first modern study. Subsequently the shroud was made available for scientific examination, first in 1969 and 1973 by a committee appointed by Cardinal Michele Pellegrino¹ and then again in 1978 by the Shroud of Turin Research Project (STURP)². Even for the first investigation, there was a possibility of using radiocarbon dating to determine the age of the linen from which the shroud was woven. The size of the sample then required, however, was ~500 cm², which would clearly have resulted in an unacceptable amount of damage, and it was not until the development in the 1970s of small gas-counters and accelerator-mass-spectrometry techniques (AMS), requiring samples of only a few square centimetres, that radiocarbon dating of the shroud became a real possibility.

To confirm the feasibility of dating the shroud by these methods an intercomparison, involving four AMS and two small gas-counter radiocarbon laboratories and the dating of three known-age textile samples, was coordinated by the British Museum in 1983. The results of this intercomparison are reported and discussed by Burleigh *et al.*³.

Following this intercomparison, a meeting was held in Turin in September–October 1986 at which seven radiocarbon laboratories (five AMS and two small gas-counter) recommended a protocol for dating the shroud. In October 1987, the offers from three AMS laboratories (Arizona, Oxford and Zurich) were selected by the Archbishop of Turin, Pontifical Custodian of the shroud, acting on instructions from the Holy See, owner of the shroud. At the same time, the British Museum was invited to help in the certification of the samples provided and in the statistical analysis of the results. The procedures for taking the samples and treating the results were discussed by representatives of the three chosen laboratories at a meeting at the British Museum in January 1988 and their recommendations⁴ were subsequently approved by the Archbishop of Turin.

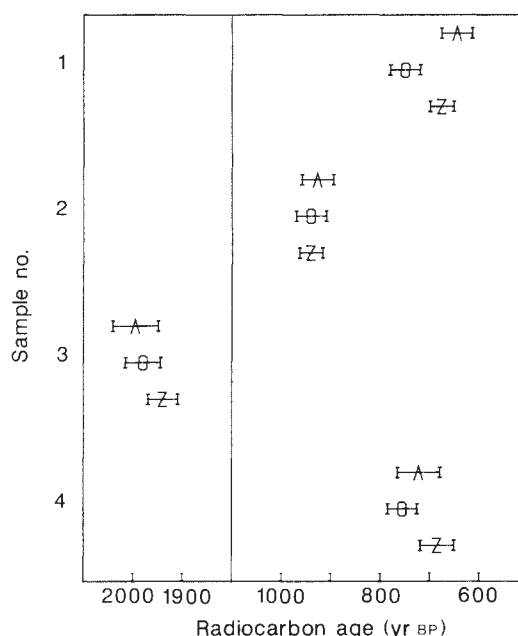


Fig. 1 Mean radiocarbon dates, with $\pm 1\sigma$ errors, of the Shroud of Turin and control samples, as supplied by the three laboratories (A, Arizona; O, Oxford; Z, Zurich) (See also Table 2.) The shroud is sample 1, and the three controls are samples 2–4. Note the break in age scale. Ages are given in yr BP (years before 1950). The age of the shroud is obtained as AD 1260–1390, with at least 95% confidence.

Removal of samples from the shroud

The sampling of the shroud took place in the Sacristy at Turin Cathedral on the morning of 21 April 1988. Among those present when the sample was cut from the shroud were Cardinal Anastasio Ballestrero (Archbishop of Turin), Professor L. Gonella (Department of Physics, Turin Polytechnic and the Archbishop's scientific adviser), two textile experts (Professor F. Testore of Department of Materials Science, Turin Polytechnic and G. Vial of Musée des Tissus and Centre International d'Étude des Textiles Anciens in Lyon), Dr M. S. Tite of the British Museum, representatives of the three radiocarbon-dating laboratories (Professor P. E. Damon, Professor D. J. Donahue, Professor E. T. Hall, Dr R. E. M. Hedges and Professor W. Woelfli) and G. Riggi, who removed the sample from the shroud.

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The shroud was separated from the backing cloth along its bottom left-hand edge and a strip ($\sim 10\text{ mm} \times 70\text{ mm}$) was cut from just above the place where a sample was previously removed in 1973 for examination. The strip came from a single site on the main body of the shroud away from any patches or charred areas. Three samples, each $\sim 50\text{ mg}$ in weight, were prepared from this strip. The samples were then taken to the adjacent Sala Capitolare where they were wrapped in aluminium foil and subsequently sealed inside numbered stainless-steel containers by the Archbishop of Turin and Dr Tite. Samples weighing 50 mg from two of the three controls were similarly packaged. The three containers containing the shroud (to be referred to as sample 1) and two control samples (samples 2 and 3) were then handed to representatives of each of the three laboratories together with a sample of the third control (sample 4), which was in the form of threads. All these operations, except for the wrapping of the samples in foil and their placing in containers, were fully documented by video film and photography.

The laboratories were not told which container held the shroud sample. Because the distinctive three-to-one herringbone twill weave of the shroud could not be matched in the controls, however, it was possible for a laboratory to identify the shroud sample. If the samples had been unravelled or shredded rather than being given to the laboratories as whole pieces of cloth, then it would have been much more difficult, but not impossible, to distinguish the shroud sample from the controls. (With unravelled or shredded samples, pretreatment cleaning would have been more difficult and wasteful.) Because the shroud had been exposed to a wide range of potential sources of contamination and because of the uniqueness of the samples available, it was decided to abandon blind-test procedures in the interests of effective sample pretreatment. But the three laboratories undertook not to compare results until after they had been transmitted to the British Museum. Also, at two laboratories (Oxford and Zurich), after combustion to gas, the samples were

recoded so that the staff making the measurements did not know the identity of the samples.

Controls

The three control samples, the approximate ages of which were made known to the laboratories, are listed below. Two were in the form of whole pieces of cloth (samples 2 and 3) and one was in the form of threads (sample 4).

Sample 2. Linen (sample QI.T/32) from a tomb excavated at Qasr Ibrîm in Nubia by Professor J. M. Plumley for the Egypt Exploration Society in 1964. On the basis of the Islamic embroidered pattern and Christian ink inscription, this linen could be dated to the eleventh to twelfth centuries AD.

Sample 3. Linen from the collection of the Department of Egyptian Antiquities at the British Museum, associated with an early second century AD mummy of Cleopatra from Thebes (EA6707). This linen was dated in the British Museum Research Laboratory using liquid scintillation counting, giving a radiocarbon age of $2,010 \pm 80\text{ yr BP}$ (BM-2558). This corresponds to a calendar age, rounded to the nearest 5 years, of 110 cal BC–AD 75 cal at the 68 per cent confidence level⁵ (where cal denotes calibrated radiocarbon dates).

Sample 4. Threads removed from the cope of St Louis d'Anjou which is held in a chapel in the Basilica of Saint-Maximin, Var, France. On the basis of the stylistic details and the historical evidence the cope could be dated at $\sim$ AD 1290–1310 (reign of King Philippe IV).

Measurement procedures

Because it was not known to what degree dirt, smoke or other contaminants might affect the linen samples, all three laboratories subdivided the samples, and subjected the pieces to several different mechanical and chemical cleaning procedures.

All laboratories examined the textile samples microscopically to identify and remove any foreign material. The Oxford group

Table 1 Basic data (individual measurements)

	Sample 1	Sample 2	Sample 3	Sample 4	Pretreatment and replication codes
Arizona	AA-3367	AA-3368	AA-3369	AA-3370	
	A1.1b* 591 $\pm$ 30	A2.1b 922 $\pm$ 48	A3.1b 1,838 $\pm$ 47	A4.1b 724 $\pm$ 42	
	A1.2b 690 $\pm$ 35	A2.2a 986 $\pm$ 56	A3.2a (1) 2,041 $\pm$ 43	A4.2a 778 $\pm$ 88	a, method a
	A1.3a 606 $\pm$ 41	A2.3a (1) 829 $\pm$ 50	A3.3a 1,960 $\pm$ 55	A4.3a (1) 764 $\pm$ 45	b, method b
	A1.4a 701 $\pm$ 33	A2.4a (2) 996 $\pm$ 38	A3.4a(2) 1,983 $\pm$ 37	A4.4a (2) 602 $\pm$ 38	(), same subsample
		A2.5b 894 $\pm$ 37	A3.5b 2,137 $\pm$ 46	A4.5b 825 $\pm$ 44	
$\delta^{13}\text{C}$ (‰)	–25.0	–23.0	–23.6	–25.0	
Oxford	2575	2574	2576	2589	
	O1.1u 795 $\pm$ 65	O2.1u 980 $\pm$ 55	O3.1u 1,955 $\pm$ 70	O4.2u 785 $\pm$ 50	u, unbleached
	O1.2b 730 $\pm$ 45	O2.1b 915 $\pm$ 55	O3.1b 1,975 $\pm$ 55	O4.2b (1) 710 $\pm$ 40	b, bleached
	O1.1b 745 $\pm$ 55	O2.2b† 925 $\pm$ 45	O3.2b 1,990 $\pm$ 50	O4.2b (2) 790 $\pm$ 45	(), same pretreatment/ run combination
$\delta^{13}\text{C}\ddagger$ (‰)	–27.0	–27.0	–27.0	–27.0	
Zurich	ETH-3883	ETH-3884	ETH-3885§	ETH-3882	
	Z1.1u 733 $\pm$ 61	Z2.1u 890 $\pm$ 59	Z3.1u 1,984 $\pm$ 50	Z4.1u 739 $\pm$ 63	
	Z1.1w 722 $\pm$ 56	Z2.1w 1,036 $\pm$ 63	Z3.2w 1,886 $\pm$ 48	Z4.1w 676 $\pm$ 60	u, ultrasonic only
	Z1.1s 635 $\pm$ 57	Z2.1s 923 $\pm$ 47	Z3.2s 1,954 $\pm$ 50	Z4.1s 760 $\pm$ 66	w, weak
	Z1.2w 639 $\pm$ 45	Z2.2w 980 $\pm$ 50		Z4.2w 646 $\pm$ 49	s, strong
	Z1.2s 679 $\pm$ 51	Z2.2s 904 $\pm$ 46		Z4.2s 660 $\pm$ 46	
$\delta^{13}\text{C}\parallel$ (‰)	–25.1	–23.6	–22.0	–25.5	

In years BP, corrected for $\delta^{13}\text{C}$ fractionation; errors at 1σ level; see text for pretreatment details.

* The identification code for each measurement shows, in order, the laboratory, sample, measurement run, pretreatment and any replication involved.

† One anomalous replicate (of 6) obtained for independent measurement O2.2b; if rejected it reduces date by 40 yr; final date quoted actually reduced by 20 yr.

‡ Measured for samples 1 and 3; assumed for samples 2 and 4.

§ The loose weave of sample Z3.1 led to its disintegration during strong and weak chemical treatments. Z3.2 was centrifuged to avoid the same loss of material.

|| Average of separate determinations by AMS.

cleaned the samples using a vacuum pipette, followed by cleaning in petroleum ether (40 °C for 1 h) to remove lipids and candlewax, for example. Zurich precleaned the samples in an ultrasonic bath. After these initial cleaning procedures, each laboratory split the samples for further treatment.

The Arizona group split each sample into four subsamples. One pair of subsamples from each textile was treated with dilute HCl, dilute NaOH and again in acid, with rinsing in between (method a). The second pair of subsamples was treated with a commercial detergent (1.5% SDS), distilled water, 0.1% HCl and another detergent (1.5% triton X-100); they were then submitted to a Soxhlet extraction with ethanol for 60 min and washed with distilled water at 70 °C in an ultrasonic bath (method b).

The Oxford group divided the precleaned samples into three. Each subsample was treated with 1M HCl (80 °C for 2 h), 1M NaOH (80 °C for 2 h) and again in acid, with rinsing in between. Two of the three samples were then bleached in NaOCl (2.5% at pH-3 for 30 min).

The Zurich group first split each ultrasonically cleaned sample in half, with the treatment of the second set of samples being deferred until the radiocarbon measurements on the first set had been completed. The first set of samples was further subdivided into three portions. One-third received no further treatment, one-third was submitted to a weak treatment with 0.5% HCl (room temperature), 0.25% NaOH (room temperature) and again in acid, with rinsing in between. The final third was given a strong treatment, using the same procedure except that hot (80 °C) 5% HCl and 2.5% NaOH were used. After the first set of measurements revealed no evidence of contamination, the second set was split into two portions, to which the weak and strong chemical treatments were applied.

All of the groups combusted the cleaned textile subsample with copper oxide in sealed tubes, then converted the resulting CO₂ to graphite targets. Arizona and Oxford converted CO₂ to CO in the presence of zinc, followed by iron-catalysed reduction to graphite, as described in Slota *et al.*⁶. Zurich used cobalt-catalysed reduction in the presence of hydrogen, as described by Vogel *et al.*^{7,8}.

Each laboratory measured the graphite targets made from the textile samples, together with appropriate standards and blanks, as a group (a run). Each laboratory performed between three and five independent measurements for each textile sample which were carried out over a time period of about one month. The results of these independent measurements (Table 1) in each case represent the average of several replicate measurements made during each run (samples are measured sequentially, the sequence being repeated several times). The specific measurement procedures for each laboratory are given by Linick *et al.*⁹ for Arizona, by Gillespie *et al.*¹⁰ for Oxford and by Suter *et al.*¹¹ for Zurich. Arizona and Oxford measured ¹⁴C/¹³C ratios by AMS and determined the ¹³C/¹²C ratios using conventional mass spectrometry. Zurich determined both ¹⁴C/¹²C and ¹³C/¹²C quasi-simultaneously using AMS only.

The conventional radiocarbon ages were all calculated using the procedures suggested by Stuiver and Polach¹², with normalization to $\delta^{13}\text{C} = -25\text{‰}$, and were accordingly reported in yr BP (years before 1950). The errors, which are quoted in Table 1 at the 1 σ level (σ is standard deviation), include the statistical (counting) error, the scatter of results for standards and blanks, and the uncertainty in the $\delta^{13}\text{C}$ determination (Arizona includes the $\delta^{13}\text{C}$ error at a later stage, when combining subsample results; Oxford errors below 40 yr are rounded up to 40).

Results

On completion of their measurements, the laboratories forwarded their results to the British Museum Research Laboratory for statistical analysis. The individual results as supplied by the laboratories are given in Table 1. Each date represents a unique combination of pretreatment and measurement run and applies

Table 2 Summary of mean radiocarbon dates and assessment of interlaboratory scatter

Sample	1	2	3	4
Arizona	646 ± 31	927 ± 32	1,995 ± 46	722 ± 43
Oxford	750 ± 30	940 ± 30	1,980 ± 35	755 ± 30
Zurich	676 ± 24	941 ± 23	1,940 ± 30	685 ± 34
Unweighted mean*	691 ± 31	936 ± 5	1,972 ± 16	721 ± 20
Weighted mean†	689 ± 16	937 ± 16	1,964 ± 20	724 ± 20
χ^2 value (2 d.f.)	6.4	0.1	1.3	2.4
Significance‡ level (%)	5	90	50	30

Dates are in yr BP. d.f., degrees of freedom.

* Standard errors based on scatter.

† Standard errors based on combined quoted errors.

‡ The probability of obtaining, by chance, a scatter among the three dates as high as that observed, under the assumption that the quoted errors reflect all sources of random variation.

to a separate subsample, except where indicated by the identification code. From these data it can be seen that, for each laboratory, there are no significant differences between the results obtained with the different cleaning procedures that each used.

The mean radiocarbon dates and associated uncertainties for the four samples, as supplied by each of the three laboratories, are listed in Table 2 and shown in Fig. 1. Also included in Table 2 are the overall unweighted and weighted means, the weights being proportional to the inverse squared errors as quoted by the laboratories. The underlying principle of the statistical analysis has been to assume that, unless there is strong evidence otherwise, the quoted errors fully reflect all sources of error and that weighted means are therefore appropriate. An initial inspection of Table 2 shows that the agreement among the three laboratories for samples 2, 3 and 4 is exceptionally good. The spread of the measurements for sample 1 is somewhat greater than would be expected from the errors quoted.

More quantitatively, to establish whether the scatter among the three laboratory means was consistent with their quoted errors, a χ^2 test was applied to the dates for each sample, in accordance with the recommended procedure of Ward and Wilson¹³. The results of this test, given in Table 2, show that it is unlikely that the errors quoted by the laboratories for sample 1 fully reflect the overall scatter. The errors might still reflect the uncertainties in the three dates relative to one another, but in the absence of direct evidence on this, it was decided to give the three dates for sample 1 equal weight in determining the final mean, and to estimate the uncertainty in that mean from the scatter of results.

As shown in Table 2, the unweighted mean of the radiocarbon age of sample 1 and its uncertainty are 691 ± 31 yr BP. The confidence limits for sample 1 were obtained by multiplying the uncertainty by t_d , the value of a Student's t distribution with d degrees of freedom at the appropriate probability level. The value of d , which lies between the inter- and intra-laboratory degrees of freedom—that is, between 2 and 9—was estimated at 5 on the basis of an analysis of variance on the 12 individual measurements supplied by the laboratories¹⁴. Individual measurements from a particular laboratory were weighted according to their inverse squared errors, but the contributions from different laboratories were weighted equally, thus ensuring consistency with Table 2. Thus for sample 1, where the error has been estimated from the scatter, ~68% and 95% confidence limits for the true radiocarbon date were found from the 1.1 σ and 2.6 σ errors about the unweighted mean respectively, the multiplying factors being obtained from standard tables of the t_s distribution. However, for samples 2, 3 and 4, the limits were obtained in the usual way from 1 σ and 2 σ quoted errors about the weighted means, assuming normality.

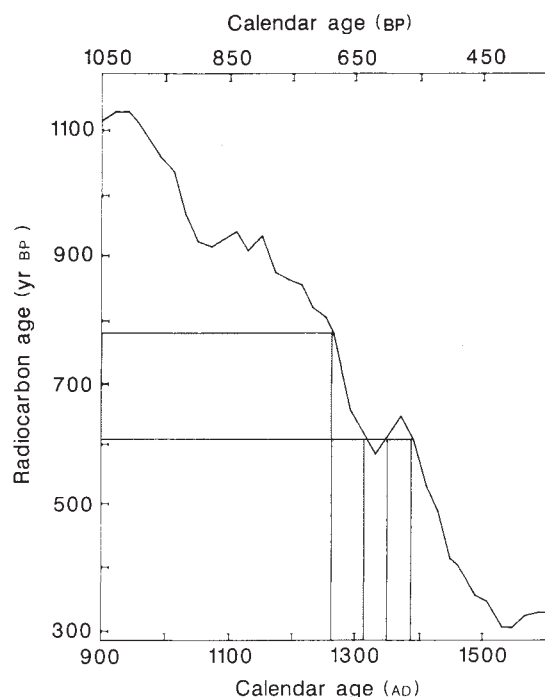


Fig. 2 Calibration of the overall mean radiocarbon date for sample 1 (the Shroud of Turin) using the 'intercept' method. (See also Table 3.) Calibration is necessary because of natural variations in atmospheric ^{14}C . The calibration curve for the relevant period is that of Stuiver and Pearson⁵, a portion of which is illustrated. The uncertainty in the calibration curve has been combined with the error in the mean radiocarbon date, giving the 95% confidence limits on the radiocarbon scale; the error envelope on the curve has therefore been omitted from the diagram. The stippled areas show how the 95% confidence limits are transformed from the radiocarbon to the calendar scale.

The calendar-age ranges which correspond to the radiocarbon confidence limits are shown in Table 3. These were determined from the high-precision curve of Stuiver and Pearson⁵ based on dendrochronological dating. Method A (the intercept method) in revision 2.0 of the University of Washington Quaternary Isotope Laboratory Radiocarbon Calibration Program¹⁵ was used. In this method, the error in the calibration curve is first incorporated into the radiocarbon error, thus widening the limits on the radiocarbon scale slightly; calendar ages are then found that correspond to these limits, without transforming the complete probability distribution of radiocarbon dates. No additional uncertainty has been added to take account of the short growth period of textile samples. There is little published guidance on this, although it has been suggested that 15 years should be added in quadrature to the overall uncertainty in the radiocarbon date for samples of growth period less than one year, such as linen. In general, such additional uncertainty would widen the 95% calendar limits by ~2–4 years at either end, except for sample 3 where the 9 cal BC limit would be changed to 34 cal BC.

The 95% limits for the shroud are also illustrated in Fig. 2, where it is apparent that the calibration of the radiocarbon date for sample 1 gives a double range. The correct transformation of probability distributions from the radiocarbon to the calendar scale is still subject to debate, there being two different methods of dealing with multiple intercepts. However, both methods agree that the major probability peak lies in the earlier of the two ranges, in the 68% range at the end of the thirteenth century. Sample 4 has a very narrow calendar range: this is due to the steep slope in the calibration curve at this point, and is an occasional instance of calibration reducing rather than increas-

Table 3 Calibrated date ranges at the 68% and 95% confidence levels

Sample	Mean date (yr BP)		Calendar date ranges
1*	691 ± 31	68%	AD 1273–1288
		95%	AD 1262–1312, 1353–1384 cal
2†	937 ± 16	68%	AD 1032–1048, 1089–1119, 1142–1154 cal
		95%	AD 1026–1160 cal
3†	1,964 ± 20‡	68%	AD 11–64 cal
		95%	9 cal BC–AD 78 cal
4†	724 ± 20	68%	AD 1268–1278 cal
		95%	AD 1263–1283 cal

* Confidence limits on the radiocarbon scale found from the standard error on the unweighted mean, assuming a t_5 distribution (multiplying factors 1.1 and 2.6 for 68% and 95% limits respectively). Standard error estimated from scatter.

† Confidence limits on the radiocarbon scale found from the standard error on the weighted mean, assuming a normal distribution (multiplying factors 1 and 2 for 68% and 95% limits respectively). Standard error computed from quoted errors.

‡ Date by conventional radiocarbon dating at the British Museum: 2010 ± 80 yr BP (BM-2558).

ing a confidence range. Sample 3 compares well with the date obtained by conventional radiocarbon dating; there is no evidence for a difference between the two results. The dates for samples 2 and 4 agree with the historical evidence, which places them in the eleventh to twelfth centuries and late thirteenth/early fourteenth centuries AD respectively.

The results, together with the statistical assessment of the data prepared in the British Museum, were forwarded to Professor Bray of the Istituto di Metrologia 'G. Colonetti', Turin, for his comments. He confirmed that the results of the three laboratories were mutually compatible, and that, on the evidence submitted, none of the mean results was questionable.

Conclusions

The results of radiocarbon measurements at Arizona, Oxford and Zurich yield a calibrated calendar age range with at least 95% confidence for the linen of the Shroud of Turin of AD 1260–1390 (rounded down/up to nearest 10 yr). These results therefore provide conclusive evidence that the linen of the Shroud of Turin is mediaeval.

The results of radiocarbon measurements from the three laboratories on four textile samples, a total of twelve data sets, show that none of the measurements differs from its appropriate mean value by more than two standard deviations. The results for the three control samples agree well with previous radiocarbon measurements and/or historical dates.

We thank Cardinal Anastasio Ballestrero for allowing us access to the shroud, Professor L. Gonella for his help and support throughout the project and Professor A. Bray for commenting on our statistical assessment of the data. We also thank Miss E. Crowfoot, T. G. H. James, Dr J. Evin, M. Prevost-Macillacy, G. Vial, the Mayor of Saint-Maximin and the Egypt Exploration Society for assistance in obtaining the three known-age control samples. Oxford thank P. H. South (Precision Processes (Textiles) Ltd, Derby) for examining and identifying the cotton found on the shroud sample; R. L. Otlet (Isotopes Measurement Laboratory, AERE, Harwell) for stable isotope ratio measurements on two samples; J. Henderson and the Department of Geology, Oxford Polytechnic for undertaking scanning electron microscopy, and SERC for the Special Research Grant which provided the primary support for the Radiocarbon Accelerator Unit. Zurich thank the Paul Scherrer Institut (PSI, CH-5234 Villigen) for technical and financial support. The AMS Programme at Arizona is partially supported by a grant from the NSF.

Received 5 December 1988; accepted 18 January 1989.

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Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1

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The crystal structure of the protease of the human immunodeficiency virus type 1 (HIV-1), which releases structural proteins and enzymes from viral polyprotein products, has been determined to 3 Å resolution. Large regions of the protease dimer, including the active site, have structural homology to the family of microbial aspartyl proteases. The structure suggests a mechanism for the autoproteolytic release of protease and a role in the control of virus maturation.

THE HIV-1 retrovirus has been shown to be the causative agent in AIDS^{1,2}. In the course of viral replication, many retroviral structural proteins and enzymes are initially translated as polyproteins which undergo enzymatic cleavage to generate the functional proteins found in mature virions³. Genetic and biochemical studies have demonstrated that a virally encoded protease is responsible for the release of structural proteins from the *gag* gene product, and for the release of protease (implying autoproteolysis), reverse transcriptase and integrase from the *gag-pol* fusion protein⁴⁻⁸. Analysis of the sequences of retroviral proteases led to the suggestion that these enzymes were members of the aspartyl protease family, on the basis of the observed conservation of a characteristic Asp-Thr-Gly active-site sequence⁹. HIV-1 protease was found to be inhibited *in vitro* by pepstatin¹⁰⁻¹², a general inhibitor of aspartyl proteases, whose action operationally defines members of this class of enzymes¹³. Furthermore, single amino-acid substitutions of the HIV-1 protease sequence in which the highly conserved Asp 25 was converted to Asn^{12,14}, Thr¹⁰ or Ala¹⁵ resulted in the elimination of protease activity. Upon transfection into mammalian cells, a provirus containing one of these mutations (Asn 25) was shown to generate non-infectious virions containing unprocessed polyprotein¹⁴. Consequently, we have focused on HIV-1 protease as a therapeutic target, and have now determined the structure of this enzyme to accelerate the rational design of protease inhibitors as potential agents in the control of AIDS.

HIV-1 protease, with only 99 amino-acid residues¹⁶⁻¹⁹, is the smallest of the retroviral proteases, and is much smaller than the microbial and mammalian aspartyl proteases, each of which contains approximately 325 residues. These pepsin-like enzymes contain homologous N- and C-terminal domains that may have evolved from a primordial gene duplication and fusion event^{20,21}. The domains, which are related by an approximate twofold axis

of symmetry, each contribute one Asp-Thr-Gly triad to the pseudo-symmetric active site. We show here that in HIV-1 protease, a strict crystallographic twofold relationship exists, in which each monomer contributes one Asp-Thr-Gly sequence to a truly symmetric active site. The structure of this active site, including the interaction of the catalytic aspartic acid residues across the twofold axis, closely resembles the active site of the pepsin-like aspartyl proteases.

Structure of HIV-1 protease

The isolation and crystallization of the HIV-1 protease have been described elsewhere²². Details of the structure determination are given in Table 1 legend. C_α coordinates will be deposited with the Brookhaven Protein Data Bank²³.

The tertiary structure of the HIV-1 protease monomer contains exclusively β -sheet, turn and extended polypeptide structural elements. Schematic descriptions of the protease structure are presented in Fig. 1. In the 3.0 Å multiple isomorphous replacement (MIR) electron density map from which this structure was derived, strong, continuous electron density is observed for all but the first five residues at the N terminal (Pro 1 to Leu 5). Chemical sequence analysis of washed crystals, however, confirmed the presence of the correct N-terminal sequence, beginning with Pro 1 (J. Rodkey, unpublished results). We conclude that the first five residues are present in our crystallized protein, but that they are disordered crystallographically, probably because of an inherent flexibility (see below). It should be noted that our current model leaves no regions of electron density in the MIR map unassigned.

In this crystal structure, the two monomer components of each HIV-1 protease dimer are related by a crystallographic twofold axis of symmetry. The overall shape of the dimer is very asymmetric; it can be approximated by an oblate ellipsoid, roughly 55 by 35 by 25 Å. To simplify the description of the dimer, the two monomers will arbitrarily be denoted A and B, even though they are crystallographically equivalent (Fig. 2). The dimer is stabilized by two sets of antiparallel interactions,

‡ Sadly, Dr Irving S. Sigal was killed in the destruction of Pan Am Flight 103 over Lockerbie, Scotland on 20 December 1988.

Table 1 HIV-1 protease heavy-atom refinement parameters

Derivative	Site	X	Y	Z	Occupancy*	B(Å ²)			
(C ₆ H ₅)Hg(C ₂ H ₃ O ₂)	1	0.046	0.026	0.052	80.0	54.0			
	2	0.165	0.962	0.101	33.8	133.0			
	3	0.108	0.005	0.056	8.7	1.7			
Pb(NO ₃) ₂	1	0.859	0.849	−0.003	17.8	36.0			
Resolution (Å)									
	11.7	8.3	6.4	5.2	4.4	3.8	3.4	3.0	Total
Native									
Observations (no.)	50	104	171	264	369	474	490	177	2,099
Figure of merit	0.93	0.93	0.88	0.81	0.79	0.65	0.47	0.38	0.67
(C ₆ H ₅)Hg(C ₂ H ₃ O ₂)									
Phasing power†	4.74	5.31	5.57	3.18	2.65	2.35	2.08	1.78	3.03
R _{Cullis} ‡	0.28	0.24	0.30	0.38	0.51	0.52	0.75	0.69	0.43
Pb(NO ₃) ₂									
Phasing power†	2.10	2.65	3.03	2.02	1.43	1.48	0.00	0.00	1.86
R _{Cullis} ‡	0.45	0.41	0.35	0.51	0.62	0.65	0.00	0.00	0.51

HIV-1 protease from the NY5 isolate³⁶ (Pro 1 to Phe 99) was crystallized in space group $P4_12_12_1$; the crystals have cell constants $a = b = 50.29 \text{ \AA}$ and $c = 106.80 \text{ \AA}$. The heavy-atom derivatives used in the structure determination were formed by soaking the crystals in 1 mM solutions of either phenyl mercury acetate or lead nitrate for up to 12 hours at 4°C. Before data collection, the crystals were washed and backsoaked for at least 2 hours in the crystallization buffer without dithiothreitol. Diffraction data were recorded beyond 3.0 Å resolution using a Nicolet X-ray area detector and CuK α radiation from a Rigaku RU-200 rotating anode X-ray source operating at 50 kV and 60 mA. Each data set was measured from a single crystal as a series of 5 min, 0.25° oscillation frames. A total of 440 individual frames, the equivalent of a 110° sweep, was collected and processed using the XENGEN processing software³⁷. The native data set had an average sixfold redundancy, with an overall R_{sym} of 0.063 to 3.0 Å resolution. The derivative data sets were measured and processed to preserve the signal that results from anomalous dispersion effects. Each derivative data set had an average threefold redundancy, with an overall R_{sym} of 0.062 for the phenyl mercury acetate derivative and 0.076 for the lead nitrate derivative. Unless otherwise stated, all subsequent calculations were performed using the computer program package PROTEIN³⁸. Initial heavy-atom positions were determined from difference Patterson maps using a vector verification technique. $P4_12_12_1$ was shown to be the correct space group from difference Fourier calculations using the two derivative data sets. MIR refinement of the heavy-atom sites gave the values shown above. A contoured mini-map of electron density was computed using native structure factor amplitudes and MIR phases, and a tracing of the polypeptide chain was derived from this map. A full set of coordinates was created from C_α guide points obtained from the mini-map and the known protein sequence³⁶. These coordinates were adjusted into the MIR electron density using the graphics program FRODO³⁹. Coordinates were refined using the constrained/restrained refinement program CORELS⁴⁰ from a starting R -factor of 0.485 to an R -factor of 0.370 at 3.0 Å resolution in four steps: the fitted coordinates were regularized, the model was refined as a rigid body, individual amino acids were refined as constrained objects, and side-chain dihedral angles were refined. These coordinates are the basis for the discussion of the HIV-1 protease structure presented here. We have initiated refinement using the program XPLOR⁴¹ followed by refinement using the program PROLSQ⁴². Currently the R -factor without the inclusion of solvent is 0.25 for a model with reasonable geometry. Further rebuilding of the model and refinement are in progress.

* Normalized to 80 electrons for the highest occupancy mercury site.

† Phasing power = $\{\sum F_H^2 / \sum (F_{PH}(\text{obs}) - F_{PH}(\text{calc}))^2\}^{1/2}$ and $R_{\text{Cullis}}^\ddagger = \sum |F_{PH}(\text{obs}) - F_{PH}(\text{calc})| / \sum |F_{PH}(\text{obs}) + F_P(\text{obs})|$ (for centric reflections) where F_P , F_H and F_{PH} are the structure factor amplitudes of the parent molecule, heavy atom and derivative, respectively.

involving residues Asp A25, Thr A26 and Gly A27 with Gly B27, Thr B26 and Asp B25, and, similarly, Thr A91, Gln A92 and Ile A93 with Ile B93, Gln B92 and Thr B91. There is an additional kinked antiparallel interaction involving Gln A7, Arg A8 and Pro A9 and Asn B98, Leu B97 and Thr B96, although these residues seem to be too far apart for cross-chain hydrogen bonding at this stage of the analysis. Side chains also seem to contribute to dimer stabilization. In particular, the position and orientation of both Asp A25 (and B25) and Thr A26 (and B26) are consistent with the highly conserved hydrogen-bonding scheme observed in aspartyl proteases of known structure²⁴⁻²⁶. Hydrophobic packing of side chains (involving, for instance, Trp A6 with Phe B99 and Ile A50 with Ile B50) may also contribute to the stabilization of the quaternary structure. Although the HIV-1 protease sequence contains two cysteine residues, at positions 67 and 95, these residues do not form disulphide linkages in the structure, either within or between monomers.

Protease structures compared

The HIV-1 protease structure was systematically compared to the structures of the three aspartyl proteases, all of microbial origin, for which coordinates are available from the Protein Data Bank²³: rhizopuspepsin²⁴, penicillopepsin²⁵ and endothiapepsin²⁶. From this analysis we concluded firstly, that large regions of the HIV-1 protease, including residues in the protease active site, have structural analogues in the microbial aspartyl proteases, and secondly, that a four-stranded β -sheet

in the HIV-1 protease dimer (two strands from each monomer) differs in orientation and connectivity from the corresponding six-stranded β -sheet (three strands from each domain) observed in the microbial aspartyl proteases.

Toh and coworkers⁹ noted homology between portions of the amino-acid sequences of retroviral proteases and the active-site sequences of pepsin-like aspartyl proteases. HIV-1 protease has structural homology corresponding to this sequence homology. The C_α positions of the 15 residues which were predicted to be homologous by Toh and coworkers, including the catalytic Asp-Thr-Gly triad, can be aligned with a r.m.s. deviation of 1.32 Å or less (Table 2) with corresponding residues in rhizopuspepsin, penicillopepsin and endothiapepsin.

This structural homology between HIV-1 protease and the microbial proteases extends beyond the active sites, as shown in Table 2. When a monomer of HIV-1 protease is compared to the N-terminal domains of the microbial proteases, 47 residues can be aligned within the same r.m.s. tolerance as found for residues in the active-site loops. The region of the N-terminal domain referred to as the flap because of its apparent flexibility was specifically excluded from this alignment, however. Residues Lys 43 to Gln 58 of HIV-1 protease constitute an analogous structure. The position of the flap with respect to the body of the HIV-1 protease structure resembles most closely the position of the equivalent residues in penicillopepsin. In that structure, residues in the flap participate in interactions with symmetry-related molecules in the crystallographic unit

When the HIV-1 protease dimer is aligned with the intact microbial proteases, the r.m.s. deviation of the fit increases significantly (Table 2). This increased deviation reflects the fact that although the HIV-1 protease dimer is truly twofold sym-

The C $_{\alpha}$ coordinates of the residues in the current model of the HIV-1 protease structure were aligned with the C $_{\alpha}$ coordinates of each aspartyl protease by a least-squares rigid-body procedure. In each comparison, the r.m.s. deviation of the aligned C $_{\alpha}$ positions is given as a measure of the quality of the fit. In the comparison with intact aspartyl proteases, the HIV-1 protease dimer was treated as a rigid body, as were the pepsin-like aspartyl proteases. The indicated residues in each monomer (denoted arbitrarily as A and B) of the rigid dimer were simultaneously aligned with residues in the N- and C-terminal aspartyl protease domains.

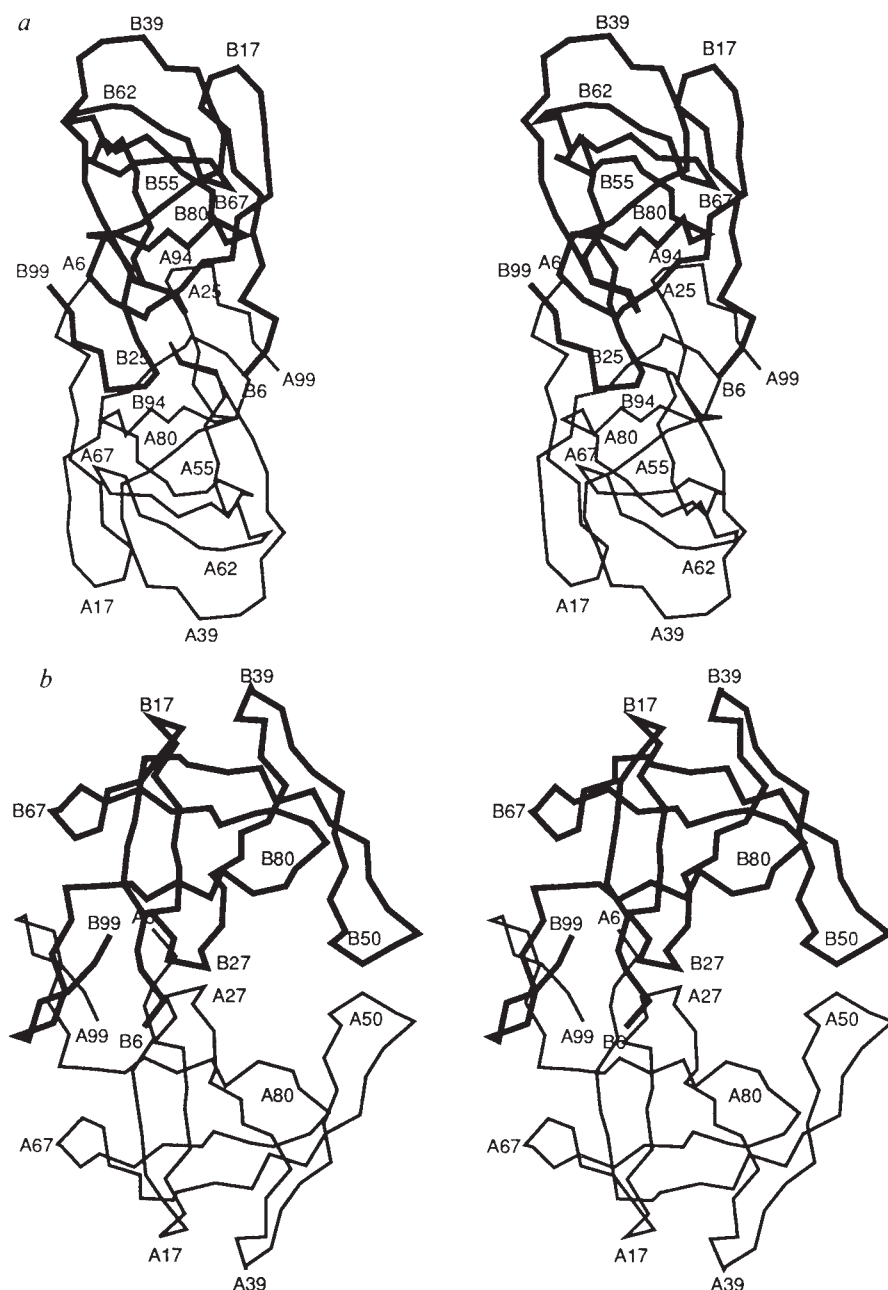


Fig. 2 A C_{α} representation of the structure of residues 6–99 of the HIV-1 protease dimer, with one monomer (arbitrarily labelled A) drawn in thin lines and the second monomer (arbitrarily labelled B) drawn in thick lines. *a*, The view is along the twofold symmetry axis of the protease dimer. *b*, The view represents a rotation of 90° about the vertical axis of the view of (*a*).

metric, the twofold axis that relates the N- and C-terminal domains of microbial aspartyl proteases is only approximate. (The relationship in penicillopepsin has been described²⁰ as a rotation of 174.6° and a translation of $1.0 \text{ \AA}$.) As Fig. 3 illustrates, the most striking difference between the superimposed structures occurs in the orientation and connectivity of the β -sheet formed by residues at the N and C terminals (note that these regions are not included in the rigid body superposition).

The topological homology between an HIV-1 protease monomer and the N- and C-terminal domains of the microbial aspartyl proteases involves the same residues that are homologous between the two domains. There are 85 pairs of residues in the N- and C-terminal domains of rhizopuspepsin that can be aligned with an r.m.s. deviation for the C_{α} positions of $2.3 \text{ \AA}$ ²⁴. If we exclude residues in the six-stranded sheet from this list, 57 pairs of residues remain. All 47 of the residues used in the N-terminal alignment of Table 2 are in this set of domain-equivalent residues, as are all 29 of the residues used in the C-terminal alignment.

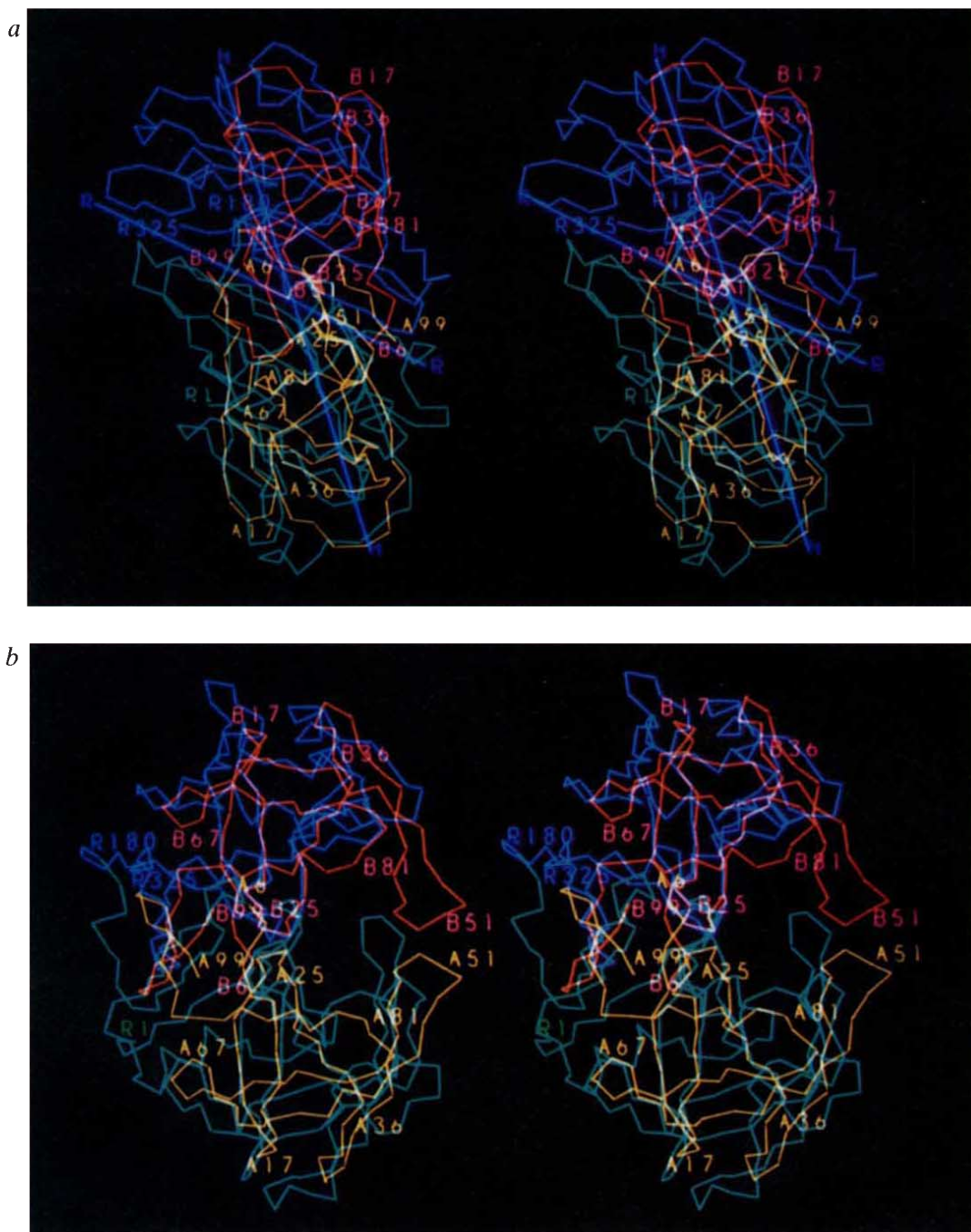
We have also compared the structure of the HIV-1 protease reported here to the model for the structure proposed by Pearl

and Taylor²⁷. To the extent that the X-ray structure is similar to the microbial aspartyl protease structures, from which their model was derived, it would seem that their predictions are reasonable. There are significant differences between the structure and their model, however. These differences include the topology of the N- and C-terminal regions and the prediction of helical structure near the C terminal (compare their Fig. 2 with our Fig. 1*a*).

Release from the *gag-pol* product

The HIV-1 protease monomer is synthesized as part of a *gag-pol* fusion product which results from an infrequent ribosomal frame shift from the *gag* to the *pol* reading frames²⁸. Evidence suggests that the protease can autocatalytically release itself from the *gag-pol* polypeptide^{7,16,29}. The structure of the HIV-1 protease dimer presented here suggests a mechanism by which autoproteolysis at its N-terminal connections to the polypeptide may occur. From the observed position of residue Trp 6 in the structure and the implied flexibility of the five N-terminal residues (see above), it seems that the scissile Phe-Pro bonds could be positioned in the protease active site for cleavage. The

Fig. 3 A comparison of the structure of the HIV-1 protease dimer with rhizopuspepsin. 47 residues in monomer A of HIV-1 protease (gold) have been aligned with the corresponding residues in the N-terminal domain of rhizopuspepsin (green); 29 residues in monomer B of the protease (red) have been aligned with residues in the C-terminal domain (blue). The views in (a) and (b) are as in Fig. 2. The lines in Fig. 3a emphasize the approximate 50° difference in orientation of the multistranded β -sheets in the two structures (R indicates rhizopuspepsin, H indicates HIV-1 protease). The connectivity of the sheets is also different. The N-terminal domain of rhizopuspepsin contributes strands to the half of the sheet that lies to the lower left of the approximate dimer axis when viewed down that axis; the C-terminal domain contributes strands that lie to the upper right. In contrast, monomer A of the HIV-1 protease dimer, which has been aligned with the N-terminal domain of rhizopuspepsin, contributes strands to the half of the sheet that lies to the right of the dimer axis; monomer B, aligned with the C-terminal domain, contributes strands that lie to the left.



pol-encoded residues between the frameshift site and the protease have no known structural or catalytic function. They may, in fact, serve as a flexible linker to facilitate the N-terminal cleavage process. All of this implies that the N-terminal clips needed to release the protease could be accomplished intramolecularly.

Analogous C-terminal cleavages are more difficult to envisage because this sort of cleavage would require the unravelling of a substantial portion of the structure to position in the active site the bond connecting the C-terminal Phe 99 of HIV-1 protease to the directly adjacent N-terminal Pro 1 residue of the much larger reverse transcriptase. We suggest that the cleavage at the C terminal can be carried out intermolecularly by a second HIV-1 protease dimer.

Control of HIV-1 assembly

The process of viral assembly takes place at the plasma membrane of the host cell. Both the *gag* and *gag-pol* products migrate

there and become anchored to the cytoplasmic side by a covalent, N-terminal-linked myristic acid^{30,31}. Electron micrographs of the virus assembly process suggest that the polyproteins aggregate, the membrane bulges outward and nascent viral particles bud from the plasma membrane³². The generation of mature viral enzymes and structural proteins from these polyproteins does not seem to occur until after the virion has budded^{14,32}. The conversion of the immature virion to the morphologically distinct, infectious entity requires the participation of HIV-1 protease¹⁴.

By analogy with the pepsin-like aspartyl proteases, only a dimer of HIV-1 protease would be catalytically competent. The requirement that dimers form before proteolysis can occur indicates a possible role for HIV-1 protease in the control of virus maturation. If an active protease dimer were to form before the nascent virus had budded from the host cell, autoproteolysis at the N terminal could occur. The symmetric nature of the protease active site allows this clip to occur for each of the two anchoring

gag-pol products, effectively solubilizing the protein, and permitting the protease (and the C-terminal linked enzymes) to diffuse away from the plane of the membrane. This diffusion would prevent premature initiation of virus maturation. Once the nascent virus particles have budded from the cell membrane, however, autoproteolytically liberated protease could diffuse only within the isolated environment of the virus particle. A cascade of proteolytic events, liberating *gag* and *pol* structural proteins and enzymes (including additional copies of the protease itself) would be initiated. Such a proteolytic cascade in the confined space of the viral envelope would generate a burst of mature viral proteins and allow the rapid self-assembly necessary to achieve full infectivity. If this mechanism is correct, it implies that retroviral proteases, including HIV-1 protease, may be more than 'fossil' examples of an ancestral dimeric aspartyl protease²⁷.

Conclusions

Self-assembly of two identical monomers into a symmetric struc-

ture represents an elegant method for creating an active enzyme while encoding a minimal amount of genetic information. The proposed mechanisms of action of the aspartyl proteases³³⁻³⁵ require the exact symmetry observed in the HIV-1 protease dimer to be broken at some point during the binding and catalysis of intrinsically asymmetric peptidyl substrates. Because of the symmetric nature of the active site, however, the initial orientation of substrate can be either right-to-left or left-to-right in the view of Fig. 2a.

HIV-1 protease is the first of the HIV-1 proteins to have its structure determined. The close resemblance between its active sites and those of the pepsin-like aspartyl proteases, where many inhibitor complexes have been investigated crystallographically suggests that it may be possible to carry over the insights derived from these studies to the design of specific HIV-1 protease inhibitors, for use as safe and effective agents in the control of AIDS.

We thank Dr John Mertz of Cray Research Inc., Minnesota, for the refinement calculations using program XPLOR.

Received 24 November 1988; accepted 12 January 1989.

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Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria

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A nuclear encoded mitochondrial heat-shock protein hsp60 is required for the assembly into oligomeric complexes of proteins imported into the mitochondrial matrix. hsp60 is a member of the 'chaperonin' class of protein factors, which include the Escherichia coli groEL protein and the Rubisco subunit-binding protein of chloroplasts.

MOST mitochondrial proteins are encoded by nuclear genes, synthesized in the cytosol as precursor proteins and subsequently imported into the organelles (for reviews see refs 1-3). Newly synthesized precursors have to assume an 'unfolded' conformation to be translocated across the mitochondrial membranes⁴⁻⁶. Cytosolic heat-shock proteins of the hsp70 family plus an addi-

tional activity which might be an ATP-dependent 'unfoldase' may have a role in maintaining this translocation-competent conformation⁷⁻¹². Transport across the mitochondrial membranes occurs at translocation contact sites between outer and inner membranes^{13,14}. Proteolytic cleavage of presequences is performed during or after translocation by the mitochondrial

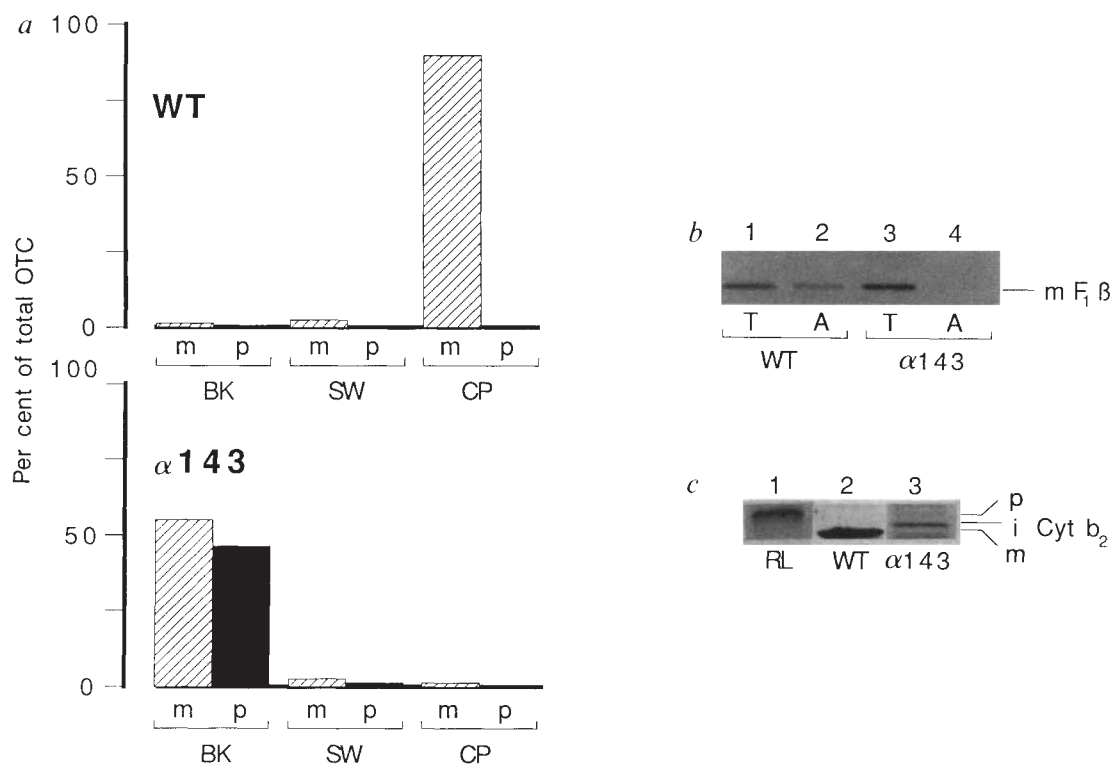


Fig. 1 Analysis of mitochondrial proteins in $\alpha 143$ cells. **a**, Defective assembly of OTC. Amounts of OTC contained in breakthrough (BK), salt wash (SW), and carbamyl phosphate elution (CP) of PALO columns are expressed as percentages of total OTC in cell extracts. p, Precursor; m, mature-sized OTC. **b**, Defective assembly of F₁ β -ATPase. Lanes 1 and 3, immunoprecipitation of F₁ β -ATPase from total amount (T) of mitochondrial fractions equivalent to the amounts analysed by chloroform extraction; lanes 2 and 4, immunoprecipitation of aqueous phase (A) of chloroform extracts. **c**, Defective maturation of cytochrome b₂. Lane 1, precursor of cytochrome b₂ immunoprecipitated after synthesis in a reticulocyte lysate (RL) directed by mRNA transcribed from the cytochrome b₂ gene cloned into pGEM3 (refs 20, 41); lane 2, mature-sized cytochrome b₂ immunoprecipitated from [³⁵S]methionine-labelled wild-type cells (WT); lane 3, intermediate-sized cytochrome b₂ immunoprecipitated from $\alpha 143$ cells.

Methods. **a**, Yeast cells were grown at 23 °C in YPEG medium until $A_{600} = 1$. Five A_{600} units of cells were resuspended in 1 ml fresh YPEG medium. After 30 min at 37 °C, galactose was added and incubation carried on for 2 h. Spheroplasts were then prepared¹⁸, extracted with 20 mM Hepes, pH 7.4/1% Lubrol, and analysed on PALO columns³⁰. Eluted fractions were precipitated with trichloroacetic acid (TCA) and analysed by SDS gel electrophoresis⁴² and immunoblotting using anti-OTC antiserum and ¹²⁵I-protein A. Fluorographs were quantified by densitometry²⁰. **b**, For *in vivo* labelling cells were grown at 23 °C in minimal galactose medium to an OD₆₀₀ of 0.5. Four A_{600} units of cells were resuspended in 2 ml fresh minimal galactose medium and shifted to 37 °C. After 2 h, 400 μ Ci [³⁵S]methionine was added and incubation carried on for an additional 30 min. The reaction was stopped by adding NaN₃ to 10 mM and 25 OD₆₀₀ units of nonlabelled cells as carrier. Spheroplasts were prepared and a crude mitochondrial fraction was isolated^{18,20}. 5×10^6 c.p.m. of wild-type and 10×10^6 c.p.m. of $\alpha 143$ mitochondrial fractions were adjusted to 100 μ l with 0.6 M sorbitol buffer and extracted with 50 μ l chloroform³¹ by vortexing for 1 min. Approximately 7.5% of total counts in wild-type and 4.5% in $\alpha 143$ were recovered in the aqueous phase. The aqueous phases were boiled in SDS-containing buffer and diluted 20-fold with TNTE (20 mM Tris pH 7.8, 150 mM NaCl, 1% Triton X-100 and 5 mM EDTA). F₁ β -ATPase was immunoprecipitated¹⁸ and the precipitates were analysed by SDS gel electrophoresis and fluorography.

processing peptidase in the matrix¹⁵⁻¹⁸. Some precursors of intermembrane-space proteins are re-translocated from the matrix back across the inner membrane by a mechanism similar to bacterial protein export^{19,20}. Finally, all imported proteins have to (re)fold and in most cases assemble into supramolecular complexes to become functionally active. Very little is known about these last stages of the import pathway.

The complex processes involved in folding and assembly of proteins imported into mitochondria might not occur spontaneously but rather might require one or more gene products. To detect such components, a bank of temperature-sensitive lethal yeast mutants was screened for a phenotype which results from deficiency in assembly of mitochondrial proteins. We describe here a nuclear mutation, *mif4*, in which subunits of mitochondrial enzymes such as subunit β of F₁-ATPase are completely translocated into the organelles, are processed to their mature-sized forms but fail to assemble into their respective supramolecular complexes. Proteins with complex presequences like cytochrome b₂ and the Rieske Fe/S protein of complex III are also affected. These proteins normally pass through the matrix on their way to the intermembrane space and they probably require interaction with protein factors during their

assembly process. In the *mif4* mutant they accumulate as incompletely processed import intermediates.

The *Mif4* gene codes for a constitutively expressed stress protein, hsp60, which is structurally related to the α -component of the chloroplast Rubisco subunit-binding protein and the *E. coli* heat-shock protein groEL^{21,22}. These two proteins have been named 'chaperonins'^{21,23} because they were proposed to chaperone oligomeric protein assembly, such as bacteriophage head assembly in *E. coli*^{24,25}, and Rubisco assembly in chloroplasts^{26,27}. Yeast hsp60 is localized in the mitochondrial matrix as a high molecular weight complex which has a distinct sedimentation behaviour on sucrose gradients. We suggest that interaction of imported proteins with this hsp60 scaffold is an essential step in conferring a conformation to imported precursor proteins which renders them competent for assembly with other subunits or for further intramitochondrial sorting.

Mutants

Mutants lacking enzymatic activity of imported mitochondrial proteins. Recently, we described a procedure for the selection of so-called *mif*-mutants (*mif*, for mitochondrial import functions), conditional yeast mutants affecting the mitochondrial

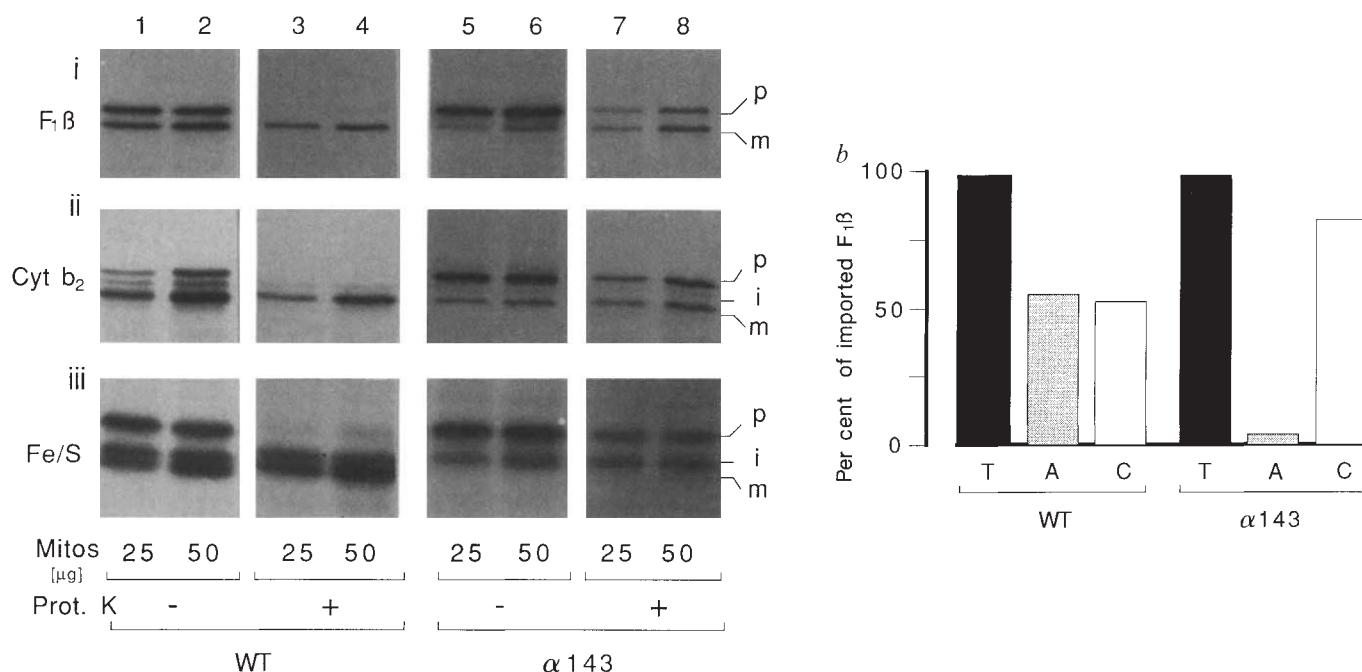


Fig. 2 Import of precursor proteins into isolated $\alpha 143$ mitochondria. *a*, Import of precursors of $F_1\beta$ (i), cytochrome b_2 (ii) and Rieske Fe/S protein (iii) into isolated mitochondria of wild-type (WT) and $\alpha 143$ mutant. Prot. K, proteinase K; p, precursor; i, intermediate-sized form; m, mature-sized form. *b*, Assembly of $F_1\beta$ imported *in vitro* into F_1 -ATPase complex. Amounts of mature-sized $F_1\beta$ contained in chloroform (C) and aqueous phases (A) are given as percentages of total $F_1\beta$ contained in 50 μg of mitochondria (T) subjected to chloroform extraction. **Methods.** Wild-type and $\alpha 143$ yeast were grown overnight in YPEG at 23 °C. Then the temperature was shifted to 37 °C for 1 h. Spheroplasts were prepared and mitochondria were isolated²⁰. Precursor proteins were synthesized in reticulocyte lysates in the presence of [³⁵S]methionine by coupled transcription/translation of the cDNAs ($F_1\beta$, Rieske Fe/S protein) or the genomic DNA (cytochrome b_2) cloned into pGEM3 using SP6 polymerase^{20,41,43}. *a*, For import *in vitro* mitochondria were incubated for 30 min at 25 °C in reticulocyte lysates diluted 5-fold with BSA buffer (3% bovine serum albumin, 80 mM KCl, 10 mM MOPS (3-[N-morpholino]propanesulphonic acid) pH 7.2) containing 1 mM NADH and 2 mM ATP. The final concentration of mitochondria in the import reactions was 25 μg (lanes 1,3,5,7) and 50 μg (lanes 2,4,6,8) in a reaction volume of 100 μl . The reactions were placed on ice and one half of each sample was treated with proteinase K (Prot. K) (20 $\mu\text{g ml}^{-1}$, final concentration) for 30 min at 0 °C (lanes 3,4 and 7,8) (ref. 20). The other halves remained on ice for the same period. Finally, mitochondria were re-isolated, solubilized in SDS-containing buffer and analysed by SDS polyacrylamide gel electrophoresis and fluorography^{20,42}. *b*, Mitochondria of wild-type and $\alpha 143$ cells (50 μg each) which had imported the radiolabelled precursor of $F_1\beta$ from a reticulocyte lysate were treated with protease as described above and subjected to chloroform extraction (see legend to Fig. 1b)³¹. Chloroform phases were dried under vacuum and aqueous phases precipitated with trichloroacetic acid (TCA) were analysed by SDS electrophoresis and fluorography. Fluorographs were quantified by densitometry.

protein import machinery¹⁸. Temperature-sensitive (ts) lethal mutants were derived by EMS-mutagenesis from a yeast strain (GalOTCRP/11) that is defective at the (cytosolic) ornithine transcarbamylase (OTC) locus (*arg3*) but contains the sequence coding for the human mitochondrial OTC (joined to an inducible yeast Gal1 promoter) integrated in its *URA3* locus. Upon expression in wild-type yeast cells, human OTC was imported into the mitochondrial matrix, processed and assembled to the enzymatically active trimer²⁸. Mutants defective in expression of OTC activity at the non-permissive temperature were analysed. This led to the identification of mutants which accumulated at non-permissive temperature the precursor of human OTC but also the precursors of endogenous yeast mitochondrial proteins. This class of mutants included the complementation groups *mif1* and *mif2* which affect the processing enhancing protein (PEP) and the mitochondrial processing peptidase (MPP), respectively, components required for proteolytic processing of precursor proteins^{16-18,29}.

In a second class of ts-lethal mutants mature-sized OTC subunits accumulated. One mutant, $\alpha 143$, was further characterized. At non-permissive temperature the cells produced mature-sized OTC subunits and OTC precursor. But, no OTC activity was detectable. When examined at the permissive temperature, $\alpha 143$ cells behaved like wild-type. A single ts mutation which did not reside in the *OTC* gene itself was responsible for this phenotype.

Mutation affects assembly of several mitochondrial proteins. To test whether OTC subunits in the $\alpha 143$ cells had assembled into

the homotrimeric enzyme an assay was carried out using the OTC substrate analogue δ -N-(phosphono-acetyl)-L-ornithine (PALO)³⁰ (Fig. 1a). Total extracts were prepared from cells grown for 2 h at 37 °C in galactose medium and were applied to columns containing PALO linked to epoxy-Sepharose. Extracts of wild-type cells contained only mature-sized OTC. Practically none of this was detected in the flow-through and wash fraction of the column, but OTC could be completely eluted with the OTC substrate carbamyl phosphate (Fig. 1a, upper panel). In contrast, with extracts of $\alpha 143$ cells both precursor and mature-sized OTC subunits were observed. They were quantitatively recovered in the flow-through (Fig. 1a, lower panel). The total contents of OTC polypeptides in wild-type and $\alpha 143$ cells were very similar. At non-permissive temperature mature-sized human OTC subunits were produced in $\alpha 143$ cells but failed to assemble into the homotrimeric, catalytically active, enzyme.

To find out whether the $\alpha 143$ mutation affects the assembly of an endogenous yeast mitochondrial protein we investigated the assembly of the β -subunit of F_1 -ATPase ($F_1\beta$) into the F_1 -ATPase complex (Fig. 1b). Our assay took advantage of the previous observation that upon treatment of a mitochondrial suspension with chloroform, assembled F_1 -ATPase complex partitions into the aqueous phase, whereas non-assembled subunits of the ATPase partition into the organic phase³¹. Cultures of $\alpha 143$ and of wild-type cells were grown at 23 °C, then shifted to 37 °C and radio-labelled with [³⁵S]methionine. Mitochondria were isolated and were subjected to chloroform extraction.

About 40–50% of total mature-sized $F_1\beta$ of wild-type mitochondria was recovered in the aqueous phase of the chloroform extraction, reflecting assembly of mature subunits into the ATPase complex (Fig. 1b, lane 2 versus 1). In contrast, virtually no mature $F_1\beta$ subunit of $\alpha 143$ mitochondria was detectable in the aqueous phase (Fig. 1b, lane 4 versus 3). We conclude that mature $F_1\beta$ subunits produced in the $\alpha 143$ mutant at the non-permissive temperature failed to assemble into the F_1 -ATPase complex.

This observed deficiency in assembly of two matrix-located proteins in the $\alpha 143$ cells led us to ask whether the import pathway of proteins passing through the matrix compartment *en route* to the intermembrane space was also affected. One example of such a protein is cytochrome b_2 . The precursor containing a bipartite targeting sequence is first completely translocated into the matrix and cleaved by the mitochondrial processing peptidase to an intermediate-sized form. This intermediate is directed back across the inner membrane by the second part of the presequence and cleaved to the mature-sized form at the outer surface of the inner membrane²⁰. When extracts of $\alpha 143$ cells grown at non-permissive temperature were analysed, essentially only the intermediate-sized form was detected (Fig. 1c). These observations could be explained either by a defect in the translocation of the precursor into the matrix space or by a conformational alteration conferred to the precursor within the matrix compartment.

Block is after membrane translocation. To decide which step of the import pathway was affected by the $\alpha 143$ mutation the same proteins whose assembly had been examined in intact cells were synthesized as radiolabelled precursors in a reticulocyte lysate and added to isolated mitochondria. Upon incubation with wild-type mitochondria prepared from cells grown at 37 °C, precursor of $F_1\beta$ was converted to its mature-sized form which was protected against externally-added protease (Fig. 2a, panel i). When import into mitochondria from $\alpha 143$ cells grown for 1 h at 37 °C was examined, again the mature-sized subunit (and the precursor) was observed to be protected from digestion by added protease. This excluded a defect in translocation of precursor proteins. To determine the state of assembly of the imported mature $F_1\beta$ subunits, the organelles were re-isolated from the import reactions and subjected to chloroform extraction (Fig. 2b). With wild-type mitochondria, more than 50% of the imported mature-sized $F_1\beta$ partitioned into the aqueous phase. In contrast, after extraction of $\alpha 143$ mitochondria virtually no mature-sized $F_1\beta$ was detected in the aqueous phase, indicating that the imported subunit had failed to assemble.

Precursor of cytochrome b_2 was also imported by $\alpha 143$ mitochondria into a protease-protected position (Fig. 2a, panel ii). In contrast to intact cells, isolated mitochondria from $\alpha 143$ cells accumulated the precursor rather than the intermediate-sized form. This might reflect kinetic differences between import reactions *in vivo* and *in vitro*. The activity of the mitochondrial processing enzyme measured in detergent extracts of $\alpha 143$ mitochondria was not reduced compared to extracts prepared from wild-type organelles (data not shown).

In a similar way, the mutation affected assembly of the Rieske Fe/S protein of complex III. After translocation into the matrix, this protein, like cytochrome b_2 , reaches a location at the outer surface of the inner membrane by re-translocation across the inner membrane. Fe/S precursor is proteolytically processed in two steps. In contrast to cytochrome b_2 , however, the second processing event takes place in the matrix before re-transport to the intermembrane space. The second processing event which removes the carboxy-terminal eight residues of the presequence is probably dependent on a conformational change of the protein induced by the formation of the iron-sulphur cluster¹⁹. Isolated $\alpha 143$ mitochondria imported the precursor of Fe/S protein into a protease protected position but were unable to process it to the mature-sized form (Fig. 2a, panel iii). Precursor and intermediate-sized Fe/S protein accumulated. In view of the results

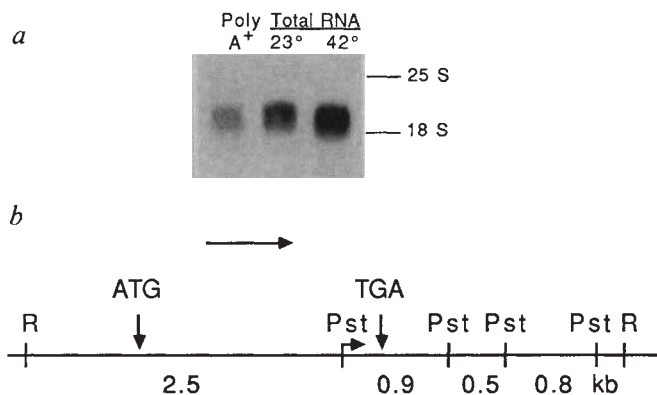


Fig. 3 Analysis of the rescuing plasmid p8. *a*, The insert of plasmid p8 recognizes a heat-inducible mRNA. 25 S and 18 S indicate migration positions of the respective yeast ribosomal RNAs. *b*, The restriction map of the p8 plasmid insert is identical to that of a λ -phage bearing the *Hsp60* gene. A 5 kilobase (kb) EcoRI fragment derived from plasmid p8 was analysed by *Pst*I digestion, and the fragment sizes compared with those of λ -Hsp60 (ref. 32). This latter clone had been isolated from a λ gt11 yeast genomic library using an antiserum directed against the 58K mitochondrial heat-shock protein of *Tetrahymena thermophila*³³. The fragment order is that indicated from sequence analysis of λ -Hsp60. A 0.9-kb *Pst*I fragment derived from p8 was sequenced in the region indicated by the horizontal arrow, and found to be identical to the one reported for the *Hsp60* gene³². The translational start and stop codons and direction of translation (long horizontal arrow) of *Hsp60* as determined from the clone are shown.

Methods. Wild-type cells were grown in YPEG medium at 23 °C to mid-log phase. Half of the culture was further incubated for 1 h at 23 °C and the other half at 42 °C. Total RNA was prepared⁴⁴. Poly(A)⁺ RNA was prepared from wild-type cells grown at 23 °C (ref. 45). RNA (10 μ g of total, 2 μ g of poly(A)⁺) was electrophoresed in a 1% agarose gel, transferred to nitrocellulose⁴⁶, and hybridized with a nick-translated 0.9-kb *Pst*I fragment derived from the p8 insert (shown in Fig. 3b).

with import of the other mitochondrial proteins analysed, it is unlikely that the $\alpha 143$ mutation affects a mitochondrial component responsible for iron-sulphur cluster formation or the second proteolytic processing of Fe/S protein.

Altogether, the data suggest that $\alpha 143$ mitochondria are defective in a component which has a role in interacting with imported precursors, conferring conformational competence required for further steps of the import pathway. Such steps may include proteolytic processing, perhaps other covalent modifications (such as iron-sulphur cluster formation), assembly into supramolecular complexes, and further membrane translocation events.

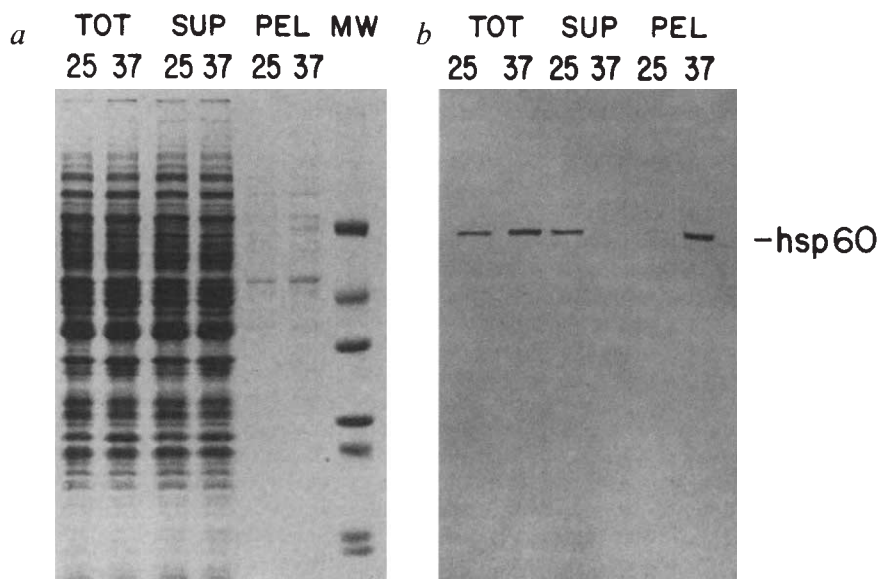
Mutation is in the gene for *hsp60*. The $\alpha 143$ strain was mated at 23 °C with a wild-type strain of the opposite mating type and the diploids were shifted to 37 °C. They exhibited a wild-type phenotype indicating that the mutation in $\alpha 143$ is recessive. The $\alpha 143$ strain was next crossed with other recessive lethal mutant strains which are defective in mitochondrial import, belonging to the complementation groups of *mif1*, *mif2*, and *mif3* (ref. 18). Diploid cells from all three crosses grew normally at 37 °C, indicating that $\alpha 143$ belongs to a different complementation group, which we designated *mif4*.

To isolate a wild-type copy of the *Mif4* gene, the haploid $\alpha 143$ strain was transformed with a library containing yeast genomic DNA fragments inserted into a centromere-bearing plasmid. DNA was prepared from colony-purified transformants growing at 37 °C and used to transform *E. coli* to ampicillin-resistance¹⁸. Plasmids were isolated and found to share several restriction fragments of inserted yeast DNA.

To test whether the rescuing plasmids might encode a heat-shock protein, an insert from one plasmid (p8) was used to probe blots containing RNA prepared from wild-type yeast

Fig. 4 Altered sedimentation of hsp60 protein in α 143 cells. Fractionation by centrifugation of detergent extracts of α 143 cells grown at 25 °C (25) or exposed to 37 °C for 2 h (37). *a*, Coomassie stained total proteins. *b*, Hsp60 as shown by immunoblotting using anti-hsp58 antiserum³³. TOT, total extracts; SUP, supernatants; PEL, pellets.

Methods. An overnight culture of α 143 cells grown at 25 °C was divided into halves. One half remained at 25 °C, whereas the other was transferred to 37 °C for 2 h. Then the cells were washed, broken by vortexing with glass beads and solubilized with 1% Triton X-100 and 0.5% deoxycholate^{22,32}. After a clarifying centrifugation at 1,000g for 5 min, the extracts were centrifuged at 15,000g for 15 min. Equivalent amounts of total extracts, 15,000g supernatants and pellets were analysed on 12.5% SDS polyacrylamide gels.



maintained at 23 °C or exposed to 42 °C for 1 h. The insert identified a poly(A)⁺ mRNA of ~1,800 nucleotides whose content in a preparation of total RNA was increased 2- to 3-fold by incubation of cells at 42 °C (Fig. 3a). It seemed possible that the *Mif4* gene encodes the heat shock protein (relative molecular mass, M_r , 60,000 (60K)) recently identified in mitochondria of yeast²². The restriction map of the insert p8 was compared with the restriction map of a λ gt11 clone containing the *Hsp60* gene³². The maps of the two cloned yeast segments overlapped precisely (Fig. 3b). A region of p8 extending over 150 base pairs (bp) was subjected to DNA sequence analysis. The sequence was identical with that from the corresponding region in *Hsp60*, which lies within the protein-coding region³² (data not shown).

To confirm that the *Hsp60* gene is the one affected by the *mif4* mutation a wild-type copy of *Hsp60* was integrated into the *Hsp60* locus in α 143 cells and mating was carried out with a wild-type strain. Following sporulation, tetrad analysis revealed that all spores were viable at 37 °C. Thus, the *mif4* mutation responsible for the observed defect of assembly of mitochondrial proteins, is in the structural gene for hsp60.

Mutant contains an altered hsp60 complex. The *Hsp60* gene is essential for the growth of yeast and encodes a precursor (~61K) that is targeted to the mitochondrial matrix, probably by an amino-terminal cleavable targeting sequence³². The mature protein bears a distinct resemblance to the 58K product of the *groEL* gene of *E. coli*, a product required for bacteriophage head assembly and essential for cell viability^{21,24}. Like *groEL*, hsp60 resides in a macromolecular complex²². Following extraction in its native state it sediments in sucrose gradients to a size position corresponding to a multimer of 12–16 identical subunits. In electron microscopic analysis of fractions containing the protein, two 7-fold symmetric ‘doughnuts’ are observed, placed one on top of the other²².

If the macromolecular complex containing hsp60 is the functionally active form, then alteration of the hsp60 component by mutation might affect the integrity of the complex. Total extracts were prepared from α 143 cells that had been either maintained at permissive temperature or shifted to 37 °C. Supernatants obtained by centrifugation at 15,000g were fractionated on sucrose gradients (data not shown). Hsp60 extracted from cells grown at 25 °C migrated as an approximately 20S particle. Analysis of extracts from α 143 cells incubated at 37 °C for 30 min revealed a much smaller portion of the total hsp60 protein at this position of the gradient. Instead, most of the product was found in the pellet of the 15,000g centrifugation. When an extract from α 143 cells which had been kept at 37 °C for 2 h was analysed, the total amount of hsp60 present increased about

2-fold but all of it was detected in the 15,000g pellet fraction (Fig. 4). In a control experiment with wild-type yeast, all of the hsp60 remained in the supernatant of the 15,000g centrifugation and migrated as 20S particles, irrespective of whether the cells were exposed to 37 °C. It is not known why hsp60 from α 143 cells exposed to 37 °C sediments into a 15,000g pellet. The hsp60 complex may have become denatured and thus precipitated. Alternatively the complex might somehow have become associated with the mitochondrial membranes, although the presence of detergents during extraction makes this unlikely. Whatever the precise alteration, we conclude that the physical properties of the protein complex containing hsp60 are affected by the mutation present in the α 143 strain.

Conclusions

Polypeptides entering the mitochondrial matrix space from the cytosol require a matrix protein, the *Mif4* gene product hsp60, for assembly and/or for further processing and sorting events. Consistent with this essential function, hsp60 is constitutively expressed. Its classification as a stress protein refers to the 2- to 3-fold induction observed in response to incubation of yeast cells at 42 °C. This response may reflect a cellular mechanism for maintaining functional conformation of mitochondrial proteins during and following heat stress.

The mechanism by which hsp60 affects the conformation of polypeptides entering the matrix space is undefined so far. The presence, however, of hsp60 in a macromolecular complex whose sedimentation properties are affected by mutation, could suggest that a ‘machinery’ is involved. Whether hsp60 is the only component of such a machinery is unknown. If the resemblance of hsp60 to the *E. coli* *groEL* protein²¹ is any indicator, however, then other proteins are likely to play a role in addition, as in *E. coli* at least one other protein, *groES*, is required for assembly of phage heads^{34,35}. Recently, the association of *groEL* with newly-synthesized, unfolded proteins has been demonstrated³⁶, suggesting that *groEL* might indeed have a function equivalent to its mitochondrial counterpart. Considering the double doughnut structure in which hsp60 apparently resides and the similar 14-subunit scaffold structure of *groEL*, we favour the idea that hsp60 may play mainly a structural role, acting as a ‘workbench’ on which folding and oligomerization is carried out by other proteins with catalytic activity. Putative catalytic components might reside either within the complex or within the soluble matrix. Included among such components could be another stress protein recently found to reside in the matrix space, belonging to the *Hsp70* gene family³⁷.

Based on the endosymbiont theory for the origin of mitochondria the utilization of hsp60 in the process of folding and assembly of imported proteins adds to the recently described evolutionarily conserved steps involved in the intramitochondrial sorting pathways of proteins of the intermembrane space^{19,20}. Although the gene for hsp60 would have emigrated to the nuclear compartment at some point after the endosymbiotic event, the acquisition of a leader peptide permitted the gene product to remain in the mitochondria. An hsp60-related function may also be present in other cellular compartments of probable endosymbiotic origin. In chloroplasts, such a function is obviously present as indicated by structural similarity of the Rubisco subunit binding-protein α -subunit to both groEL and hsp60 and by its role in the assembly of the oligomeric Rubisco complex^{21,26,27}. As defined by Ellis and colleagues^{21,23} the groEL and Rubisco subunit-binding protein belong to one class of so-called 'molecular chaperones' termed 'chaperonins'. Our findings qualify the hsp60 protein of the mitochondrial matrix as a member of the 'chaperonin' class in a functional context.

Received 22 November 1988; accepted 9 January 1989.

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The involvement of protein factors, in particular heat-shock proteins, in folding and assembly of polypeptides has been suggested previously^{10,38}. Some of these factors, such as cytosolic hsp70, may help in maintaining precursor proteins in a translocation-competent conformation. Other factors may control correct folding of newly synthesized polypeptides. The immunoglobulin heavy-chain binding protein (BiP), also known as GRP78 (refs 39, 40), might belong to this class of factors. The special function of the groEL/hsp60 family appears to be in actively catalysing the acquisition of the native conformation of oligomeric proteins. Our data strongly support the concept of protein-catalysed assembly of proteins entering the mitochondrial matrix space.

We thank A. West for help with RNA blot analysis, Dr M. Douglas for supplying anti-F₁ β antiserum and providing advice on F₁ β assembly, and S. Meier for technical assistance. This work was supported by the NIH, the Deutsche Forschungsgemeinschaft, the NSF, the Department of Energy, and by the Bernard and Jennie M. Nelson Fund. A.H. is a fellow of the John A. Hartford Foundation.

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LETTERS TO NATURE

Ultra-luminous OH maser emission from an IRAS galaxy

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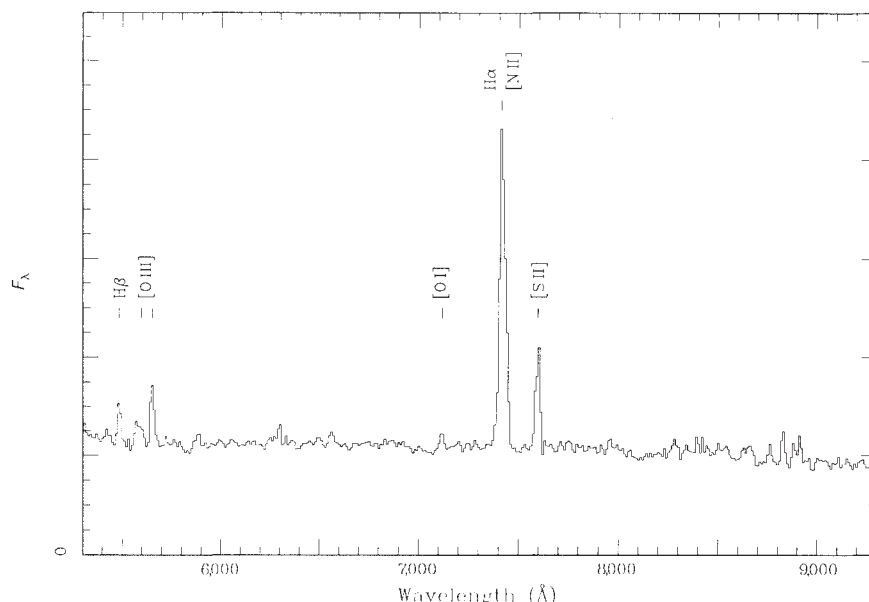
Since the discovery¹ in 1982 of intense hydroxyl (OH) maser emission from the infrared-luminous galaxy Arp 220, twenty similar sources have been found in single-dish radio searches²⁻⁷. These so-called OH megamasers are associated with infrared-luminous galaxies with far-infrared colour temperatures of about 50 K. Here we report the detection of luminous OH maser emission in the galaxy IRAS20100-4156. This galaxy, at redshift $Z = 0.129$, displays similar optical properties to Arp 220, but exceeds it in OH luminosity by well over an order of magnitude, making it the most luminous OH maser known. The existence of such luminous

megamasers increases the volume of space accessible for their study by a factor of about 50.

The generally accepted physical mechanism for OH emission in megamasers is the amplification of a centrally located continuum source by OH molecules in galactic molecular clouds⁸. The OH lines are most prominent at the main-line frequencies of 1,665 and 1,667 MHz and have velocity widths in excess of 100 km s⁻¹. Isotropic OH luminosities in the range 10²-10³ L_⊙ are typical, although a class of lower-luminosity sources (1-10 L_⊙) of somewhat smaller pumping efficiency is also known to exist (for example, NGC3690, NGC4418, IC5298).

Most searches for OH megamasers so far have been conducted using telescopes in the Northern Hemisphere. The only major search for sources in the Southern Hemisphere⁹ was limited to galaxies with 60-μm-flux densities >10 Jy. We are now extending that survey to include IRAS galaxies with fluxes <10 Jy. The candidate objects have been selected by means of the distinctive far-infrared colours¹⁰ of megamasers. Those which appear to be extragalactic, on the basis of identification from SERC sky survey films, are being searched for maser emission. Redshifts are being obtained with the Mt Stromlo 1.9-m reflector. A full description of this survey will be given on completion.

Fig. 1 Spectrum of IRAS20100-4156 taken with the Faint Object Red Spectrograph on the 3.9-m Anglo-Australian Telescope. Exposure time is 1,000 s and resolution is 20 Å. Vertical scale is flux per angstrom in arbitrary units.



Low-dispersion optical spectra of IRAS20100-4156 were obtained during the period 5–9 September 1988 using a photon-counting array on the Mt Stromlo 1.9-m telescope. A resolution of 12 Å and a wavelength coverage of 3,600–8,000 Å were used. A further spectrum, shown in Fig. 1, was taken on 27 October 1988 during PATT service time on the 3.9-m Anglo-Australian Telescope (AAT) using the Faint Object Red Spectrograph to cover the wavelength range 5,300–9,300 Å. The emission lines detected were H α /[N II], H β , [O I], [O II], [O III] and [S II]. The mean redshift of this object deduced from the AAT data, is 0.1291 ± 0.0002 . From the line ratios, the object may be classified, using the diagrams of Veilleux and Osterbrock¹¹, as a low-ionization nuclear emission-line region (LINER). There is no indication of broad Seyfert-like lines.

Radio observations of IRAS20100-4156 were taken on 28 and 29 September 1988 using the Parkes 64-m telescope with the Australia Telescope prototype broad-band receiver. Typical system temperatures were 40 K. Spectra were obtained using the Parkes digital correlator. A bandwidth of 10 MHz was used, with 256 spectral channels in each of two orthogonal linear polarizations and in each of two frequency bands. An on-source integration time of 4,200 s was obtained for the redshifted OH 1,665- and 1,667-MHz main-lines, and of 3,000 s for the redshifted OH 1,720- and 1,612-MHz satellite line frequencies. Corresponding spectra are shown in Fig. 2. The velocity scale is heliocentric and is plotted in the optical convention ($v = c\Delta\lambda/\lambda_0$). The frequency resolution is 80 kHz, equivalent to a rest-frame velocity resolution of 16 km s⁻¹ for the redshifted OH lines. Spectral interference, denoted by the gap in the spectrum in Fig. 2, was noted only for the 1,720-MHz observations.

Figure 2 reveals intense OH main-line emission with a peak flux density of ~ 200 mJy. The strongest emission peak, from the 1,667-MHz ($F=2 \rightarrow 2$) transition, occurs at $38,697 \pm 3$ km s⁻¹; there is also a secondary peak at $38,648$ km s⁻¹. A marginal detection of the 1,665-MHz ($F=1 \rightarrow 1$) transition is also observed at $39,044$ km s⁻¹, corresponding to a true velocity of $38,646$ km s⁻¹, which is close to the velocity of the secondary blueshifted 1,667-MHz peak. No features are detected at the satellite line frequencies of 1,612 and 1,720 MHz. For the parabolic baseline fitted in Fig. 2, the 1,667:1,665-MHz peak-flux-density ratio is ≥ 8 . Figure 2 also shows a pedestal of 1,667-MHz emission which extends over a velocity range of ~ 500 km s⁻¹. The total velocity range may, however, be considerably greater if, as we suspect, some of the parabolic baseline we have fitted

is actually genuine line emission. Independent main-line spectra consistently appear to show extended emission over a total velocity range up to 1,500 km s⁻¹. However, because this velocity range is comparable with the total bandwidth of the observations, further observations are required to confirm it. OH emission over a broad velocity range (300–1,000 km s⁻¹) has been found in several other luminous megamasers^{5,12,13}.

Table 1 summarizes the properties of infrared and OH emission from IRAS20100-4156. A far-infrared (1–500 μ m) luminosity of $3.0 \times 10^{12} L_{\odot}$ has been calculated¹⁴, by ignoring the 12- μ m contribution, for which only an upper limit is available, and by using a Hubble constant $H_0 = 75$ km s⁻¹ Mpc⁻¹. In common with other strong OH emitters, IRAS20100-4156 is therefore extremely luminous in the far-infrared. On the basis of the SERC sky-survey films, IRAS20100-4156 seems to be compact, with an overall diameter of 10 arcsec. It shows a double structure, with components separated by 3 arcsec, corresponding to a projected linear separation of 8 kpc. The optical morphology is similar to that of Arp 220 and to other high luminosity infrared galaxies¹⁵, and the unusual nature of this morphology suggests that, in common with most of these other galaxies, IRAS20100-4156 is a merger.

The OH luminosity of IRAS20100-4156 of $9.3 \times 10^3 L_{\odot}$, obtained by subtracting the parabolic baseline in Fig. 2, is considerably beyond the luminosity range for megamasers. It is greater than the strongest megamasers known previously (Mrk

Table 1 Properties of IRAS20100-4156

Flux densities	100 μ m	5.20 Jy
	60 μ m	5.43 Jy
	25 μ m	0.42 Jy
	12 μ m	< 0.25 Jy
	OH 1,612 MHz	< 0.03 Jy
	OH 1,665 MHz*	0.02 Jy
	OH 1,667 MHz*	0.20 Jy
	OH 1,720 MHz	< 0.03 Jy
Redshifts	Optical	0.1291 ± 0.0002
	OH 1,667 MHz (peak)	0.12908 ± 0.00001
Luminosities†	40–120 μ m‡	$2.0 \times 10^{12} L_{\odot}$
	1–500 μ m§	$3.0 \times 10^{12} L_{\odot}$
	OH 1,667 MHz*	$9.3 \times 10^3 L_{\odot}$

* Parabolic baseline fitted.

† $H_0 = 75$ km s⁻¹ Mpc⁻¹, $q_0 = 1$.

‡ Formula from ref. 19.

§ Formula from ref. 14.

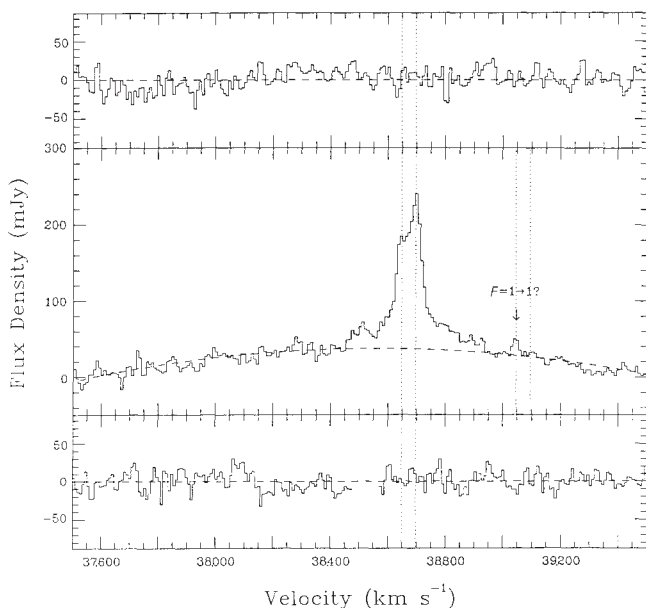


Fig. 2 Spectra of IRAS20100-4156 for the hyperfine transitions of OH at wavelength 18 cm, taken with the Parkes telescope. Velocities are in the optical convention ($c\Delta\lambda/\lambda_0$) with respect to a heliocentric rest frame. The dashed lines represent the instrumental baselines fitted to each of the three spectra. For the two satellite lines (top ($F=1\rightarrow 2$) and bottom ($F=2\rightarrow 1$)) these correspond to a constant flux density. For the main-lines (middle), a parabolic baseline is fitted. Some of the latter may, however, be genuine line emission (see text). The vertical dotted lines plotted at 38,648 and 38,697 km s^{-1} indicate the velocities of the two emission peaks in the $F=2\rightarrow 2$ transition.

231, IRAS12112+0305 and IRAS17208-0014) by almost a factor of ten. The pumping efficiency given by the flux density ratio of OH emission to 60- μm emission, $f_{\text{OH}}/f_{60} = 0.04$, is also higher than that of other megamasers. Such a high apparent efficiency provides a firm constraint for theoretical pumping models and, in particular, does not support the standard main-line pumping schemes^{16,17}. For IRAS20100-4156 there is, however, no significant deviation from the quadratic relationship between OH and far-infrared luminosity which has been postulated¹⁸ for megamasers.

The extreme luminosity of IRAS20100-4156 raises the prospect of detecting similar sources at much higher redshifts. At Parkes, sensitivity considerations limit the maximum attainable redshift to ~ 0.4 for such sources. This is also the limit at which it becomes difficult to select possible candidates from the IRAS Point Source Catalog. Although it is possible that a strong evolutionary trend could be detected with such a modest redshift range, ideally redshifts of order unity would be required to detect significant changes in number density or luminosity. Although such studies are feasible with the largest radio telescopes, and of much interest in exploring the connection between ultra-luminous infrared galaxies and quasars, the depth of existing far-infrared surveys presently precludes the detection of such high-redshift OH megamasers. Nevertheless, the detection of IRAS20100-4156 implies that, for observations with Parkes and similar radio telescopes, the volume of space accessible for the study of luminous megamasers is greater than was previously thought by a factor of ~ 50 .

Received 28 November 1988; accepted 4 January 1989.

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Detection of microvariability for BL Lacertae objects

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Large-amplitude, rapid optical variability is a well-known identifying characteristic for BL Lacertae objects ('blazars'). Although large-amplitude variations on timescales ranging from days to decades have been well documented¹, considerable controversy surrounds the nature of microvariability, that is, optical variations on timescales significantly shorter than a day. Here we report observations of BL Lacertae in which rapid changes were detected in the total optical flux from this object. These variations occurred on timescales as short as 1.5 hours. Although their structure is complex, the minimum timescale for the variations may be used to place constraints on the size of the emitting region. Assuming that these variations are produced in the vicinity of a supermassive black hole, the timescales imply a black-hole mass of $1.42 \times 10^9 M_{\odot}$. Relativistic beaming need not be invoked to account for the present luminosity of this object.

Photoelectric photometry² of BL Lac suggested the possible existence of low-amplitude, short-term variations, but subsequent observations^{3,4} failed to detect them. More recently, rapid changes have been observed^{5,6} in the polarization for BL Lac on timescales significantly shorter than a day. These results have motivated our observational programme to study, using modern CCD (charge-coupled device) detectors, the nature and indeed the existence of microvariability for a selected sample of blazars. Here we report the first results of this study for the prototype of this class of objects, BL Lac.

The observations reported here were obtained with the No.1 0.9-m telescope at Kitt Peak National Observatory equipped with a direct CCD camera and an auto-guider. The observations were made through a V-filter with an RCA CCD. Repeated exposures of 90 s each were obtained of the star field containing BL Lac. Other stars on the frame provide comparisons for use in the reduction process. The observations were reduced using the method of Howell and Jacoby⁷. Each exposure is processed through an aperture-photometry routine which reduces the data as if it were produced by a multi-star photometer. Differential magnitudes can then be calculated for any pair of stars observed on the frame. Thus simultaneous observations of the object of interest and several comparison stars allows one to remove variations arising from fluctuations in atmospheric transmission and extinction.

The light curves for observations of BL Lac obtained on 11 and 14 July 1987 are shown in Figs 1 and 2 respectively. The upper panel in each figure displays the differential magnitude between comparison star 2 and BL Lac; the lower panel displays the differential magnitude between comparison star 2 and com-

Fig. 1 Top, Differential magnitude between the variable BL Lac and comparison star 2 for the night of 11 July 1987. Bottom, Differential magnitude for comparison star 2 and comparison star 3 for the same night.

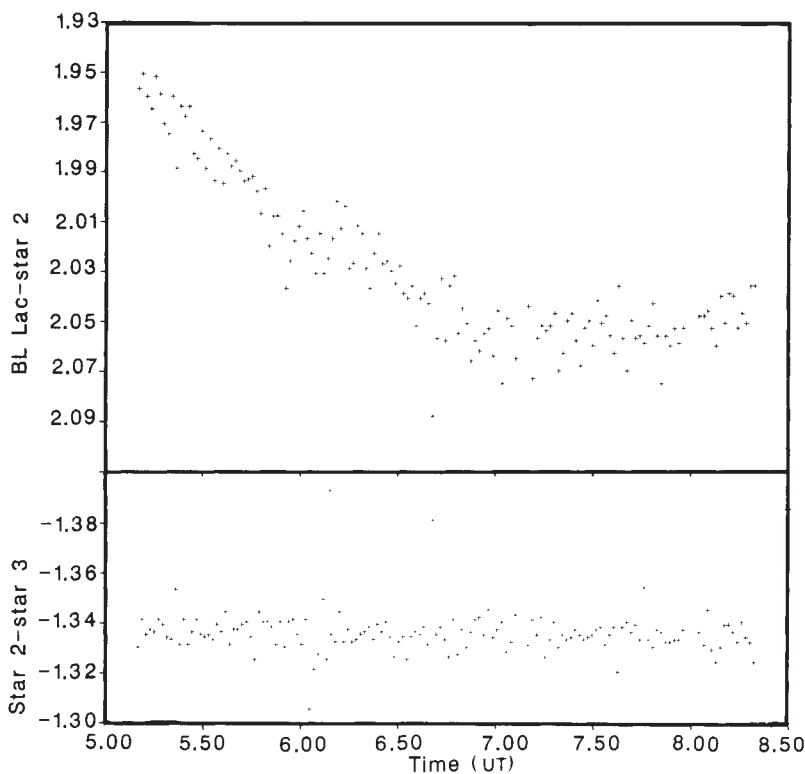
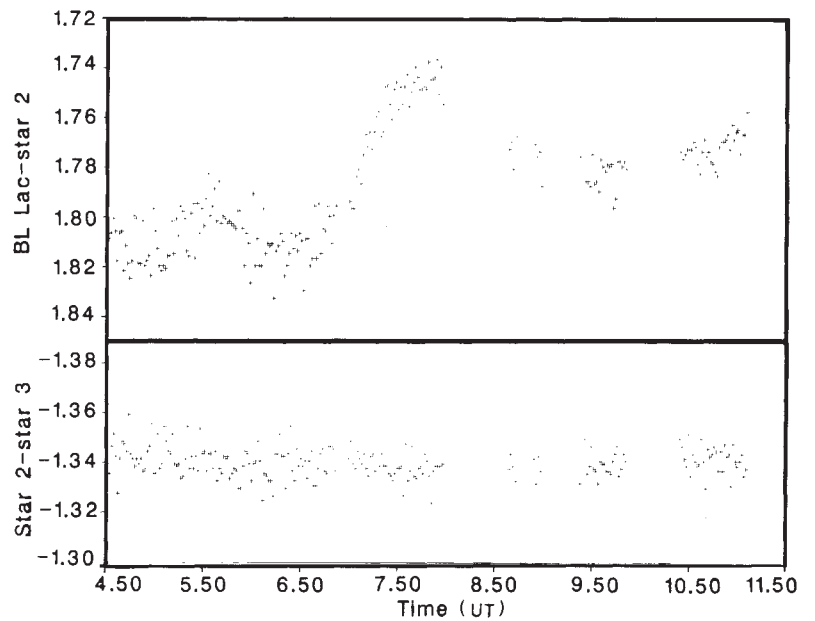


Fig. 2 Top, Differential magnitude BL Lac and comparison star 2 for the night of 14 July 1987. Bottom, Differential magnitude for comparison star 2 and comparison star 3 for the same night.

parison star 3. In each case, the scatter of the differential magnitudes in the lower panel provides an indication of the stability of the standard stars used in reducing these observations, and of the overall observational uncertainty.

On 11 July 1987 (Fig. 1) BL Lac was observed continuously for ~ 7 hours. From 04:30 to 06:30 UT, the source exhibited only mild variations, with a range of ~ 0.05 mag, and no conspicuous trends. From 06:30 to 08:00 UT, however, the microvariability of BL Lac could be characterized by a pronounced linear rise in brightness with a total change of ~ 0.12 mag. The source faded subsequently by ~ 0.04 mag, and exhibited no significant trends during the last three hours of the night.

On the night of 14 July 1987 (Fig. 2), the microvariability of BL Lac exhibited a decline of ~ 0.14 mag from 05:00 UT to

07:00 UT, but no systematic trends in brightness variations during the remainder of the night.

On 11 July 1987, when the microvariability exhibited a relatively complicated structure, BL Lac was near a brightness of $V = 15.0$ mag. This is near the middle of the range in brightness exhibited by the source in the past. It was slightly fainter (~ 0.25 mag) on 14 July 1987, when the steady decline in brightness was observed.

From a power-spectrum analysis of the time series on each of the nights we find no evidence for periodic modulations of the variations such as those reported for the blazar OJ287.

If one assumes that the microvariability detected for this object is generated in the vicinity of a supermassive black hole, one can estimate the size of the emitting region as $R = ct$,

assuming no relativistic beaming. The timescale identified from the present observations is the time during which the dramatic 0.12 mag. variation occurred on 11 July 1987. The duration of this event is approximately 1.5 hours. If one uses the timescale of this event to define a 'characteristic timescale' and one assumes that the radiation is generated close to the black hole, for example $R = 3R_s$ where $R_s = 2GM/c^2$ is the Schwarzschild radius, then the mass of the supermassive black hole, M , is given by $M = c^3 t / 6G = 1.42 \times 10^9 M_\odot$. The Eddington luminosity for a black hole of mass M is given by⁹ $L_E = 1.3 \times 10^{38} (M/M_\odot) \text{ erg s}^{-1}$ which in this case yields $L_E = 1.85 \times 10^{47} \text{ erg s}^{-1}$. For a brightness of ~ 15.0 mag, the luminosity for BL Lac is of the order of $L_v \approx 10^{45} \text{ erg s}^{-1}$.

Thus we find that although microvariability is definitely present on very short timescales, which in turn suggests a very small source region, the luminosity of BL Lac is significantly less than the Eddington luminosity, and does not require relativistic beaming for its explanation.

We thank NOAO and Lowell Observatory for generous allocations of observing time. H.R.M. is grateful for support from the Research Grant Program at Georgia State University.

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A Solar System dust ring with the Earth as its shepherd

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Bodies orbiting in the gravitational fields of galactic, solar or planetary systems often suffer dissipative forces, including tidal interactions and drag resulting from motion through a gas or from collisions with dust grains. In the early solar nebula, gas drag induces resonance trapping, which may be of importance in the early accretional growth of planets^{1–3}. By means of numerical integrations, we show here that small dust grains can be temporarily captured into exterior orbit-orbit resonances with the Earth, lasting from less than 10,000 years to more than 100,000 years. Grains with radii of 30–100 μm , orbiting in planes less than 10° from the plane of the Solar System and with orbital eccentricities of less than 0.3, are captured most easily. We argue that there should be an approximately toroidal cloud of particles, derived mostly from the asteroid belt, trapped into a variety of these exterior resonances. The cloud is mostly beyond the Earth's orbit, but includes it.

Three factors primarily motivated the present study. (1) The narrow bands observed along the ecliptic in the IRAS (Infrared Astronomical Satellite) data clearly seem to originate from dust particles in the main asteroid belt^{4,5}; indeed, asteroid collisions may provide most of the dust grains that give rise to the entire broad band of observed zodiacal thermal emission⁶. (2) Grains smaller than $\sim 100 \mu\text{m}$ in radius should successfully survive collisional destruction⁷ to spiral in, under Poynting–Robertson⁸ (P–R) and solar-wind drag, to well within the orbits of the Earth and Venus. (3) Venus and the Earth significantly perturb^{9,10} the orbits (which are shrinking under P–R drag) of dust grains that

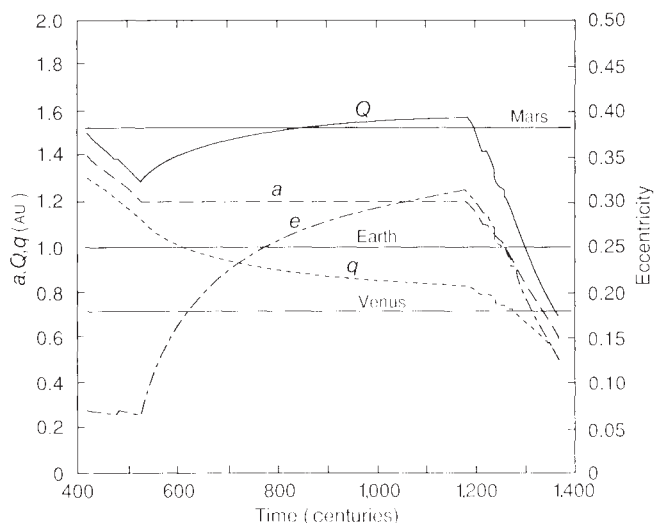


Fig. 1 Semi-major axis, a , aphelion distance, Q , perihelion distance, q , and eccentricity, e , as functions of time (in centuries) for a $30\text{-}\mu\text{m}$ radius particle started in an orbit with $a = 2.0$, $e = 0.1$ and an orbital inclination of 2.0° .

pass these planets.

Some effects from dissipative forces in the Solar System have been noted. Gas drag, for example, can trap small bodies into $j:k$ orbital-period commensurabilities with a massive perturbing body (such as an accreting planet); here j and k are integers. Orbit-period commensurabilities of the type $j:j+1$ are often observed. Gonczi *et al.*¹¹ noted that, under P–R drag, small dust grains that were initiated in orbits at the interior 2:1 resonance with Jupiter could remain trapped there for some time; they did not, however, find any evidence for dust grains drifting, under P–R drag, into that resonance and becoming trapped.

Although our conclusions are consistent with those of Gonczi *et al.*, we also obtain new and different results (reported earlier in preliminary form¹²). These new results include long-term trapping (for more than 100,000 years) into resonances exterior to the Earth's orbit, short-term trapping (for typically less than 10,000 years) into resonances exterior to the orbits of Mars and Venus, and some limited trapping into interior resonances. For calculations we used Cowell integration in cartesian coordinates using the Everhart¹³ implicit Runge–Kutta integrator with Gauss–Radau spacings. Radiation pressure is included and opposes the effect of solar gravity, with the assumption that particles behave as spherical black bodies of density 1 g cm^{-3} . The only other forces included are P–R drag and solar-wind drag, with the latter assumed to be 30% of the former. Solar-wind drag is included by multiplying by 1.3 the vector velocity term given by equation (5) in Burns *et al.*¹⁴. Many simulation runs incorporated the effect on the particles of the gravitational fields of the Sun, Venus, the Earth, Mars and Jupiter. To reduce computation, however, most runs included only the gravitational fields of the Sun, Venus and the Earth, and many were done with only those of the Sun and the Earth.

The trapping dynamics are best illustrated for the simplest case in which only the Sun, the Earth and a dust grain are involved. This case is shown in Fig. 1, where the orbital evolution of a particle of radius $30 \mu\text{m}$ is initiated with a semi-major axis, a , of 2.0 AU , an orbital eccentricity, e , of 0.1 , and an orbital inclination of 2.0° . The plot starts at 40,000 years into the simulation. For the first 52,000 years, a decreases on average, under P–R and solar-wind drag, at a rate of 7.3 cm s^{-1} . The particle is then trapped into an external 3:4 resonance with the Earth at $a = 1.2037 \text{ AU}$ for the next 65,000 years; during this trapping period, a remains approximately constant, e increases, the perihelion distance, q , decreases to less than that of the

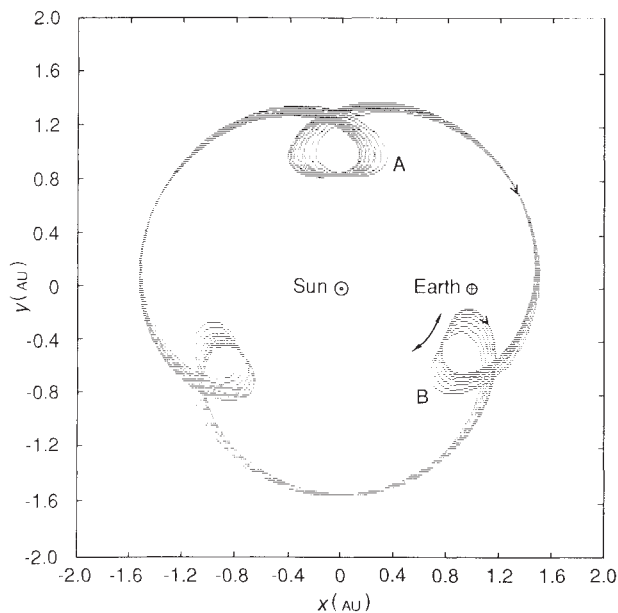


Fig. 2 The motion of the dust grain in Fig. 1, in a heliocentric coordinate frame rotating with the Earth. Shown here is the trajectory for 50 years of motion, projected onto the ecliptic, starting at 90,000 years into the simulation. The orbital motion tends to repeat itself in 220 years during quasi-stable trapping into the 3:4 external resonance with the Earth.

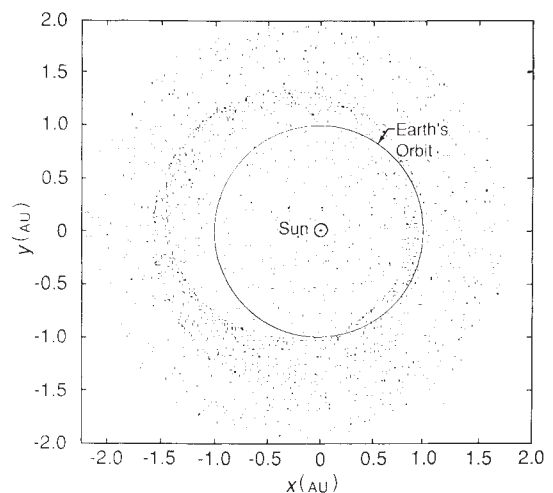


Fig. 3 The position of the particle for the orbit in Fig. 1, projected onto the ecliptic plane as a point approximately every 93 years as it evolves under gravitational and drag forces. The density enhancement arising from shepherding by the Earth is clearly evident.

Earth, and the aphelion distance, Q , increases. This trapping resonance is by no means unique: depending on the initial dust-grain orbital parameters, we have observed trapping in essentially all $j:j+1$ external resonances with the Earth from 2:3 to 13:14 (the latter for an initially circular orbit inclined at 10° to the ecliptic). We have also observed trapping into the 3:5 and 1:1 resonances. The 1:1 resonance resulted from the trapping of a $100\text{-}\mu\text{m}$ radius dust grain released from Comet Encke into a 'circulator' (rather than a horseshoe-shaped 'librator') resonance with the Earth.

Insight into the dynamics of the trapping can be obtained by transforming to a Sun-centred coordinate system rotating with the Earth's orbital motion. Figure 2 depicts particle motion, as viewed from ecliptic north, in such a coordinate system for 50 years, starting at 90,000 years into the same run as shown in Fig. 1. The Earth is shown to the right in this plot. At aphelion, the dust grain orbits more slowly around the Sun than does the Earth, and so moves in a clockwise fashion in this diagram. At perihelion, dust-grain orbital motion is faster than that of the Earth, and the grain moves in an anticlockwise direction. This pattern of motion gives rise to the three loops that are made during the four years that the particle takes to approximately retrace its path in this 3:4 resonance. The particle does not, however, exactly retrace its path on each orbit, because it is seldom precisely in the 3:4 resonance but only near it, in a quasi-stable trajectory, during the 65,000 years that it remains trapped. When the particle approaches relatively near, but trails orbitally behind, the Earth, just after particle perihelion passage, it receives a boost in orbital energy which increases its orbital semi-major axis a . If a becomes larger than the semi-major axis appropriate for the 3:4 resonance, then the dust grain has not quite completed three orbits by the time that the Earth has made four orbits. This causes the loops in the dust grain's orbit (Fig. 2) to drift in a clockwise direction. If this drift continues far enough, the gravitational downward 'kick' in the orbital energy of the dust grain can exceed the upward kick, because the dust grain now approaches closer to the Earth in loop A than in loop B. The orbital energy of the dust grain then decreases under

both the Earth's gravitational force and the P-R and solar-wind drag forces until a becomes smaller than the resonance value. The loops then drift in an anticlockwise direction, and so on in a cyclic fashion. A high-resolution plot of semi-major axis against time clearly shows that a varies continuously throughout the trapping period to values both above and below the exact 3:4 resonance value of $a = 1.2037$.

In Fig. 3, we show the position of the particle projected as a point onto the ecliptic plane every 93.4377 years. It is clearly seen that the trapping phenomenon leads to an increased particle density near, but mostly outside of, a distance of 1 AU. The particle density is not uniform in heliocentric longitude because the line of apsides of the particle's orbit rotates by only $\sim 180^\circ$, non-uniformly in time, during this particular trapping event. The orbital inclination of the particle in Figs 1, 2 and 3 remained near 2° throughout the trapping period, following which it varied rapidly to $\sim 8^\circ$.

Large grains are trapped more easily into one of the external resonances than are small grains. Our numerical experiments have not generated a resonance with Mars for particle radii less than $60\text{ }\mu\text{m}$. Large grains also tend to be trapped into resonances that have larger semi-major axes than those of small grains, and they stay trapped for longer periods of time. This can be explained by the relative rapidity with which small grains progress through a resonance trapping zone, a condition already noted for other types of trapping¹⁵. Grains with radii less than $10\text{ }\mu\text{m}$ are seldom trapped by the Earth or Venus and, when trapped, remain so only for very short periods. Grains of radius $30\text{ }\mu\text{m}$ are typically trapped exterior to the Earth for time spans of $\sim 30,000$ years, although they occasionally escape trapping altogether (even for orbits of low e and inclination). The overall physical process may be described as the 'shepherding' of dust grains into external orbit-orbit resonances by the combined perturbations of the Earth's gravity and P-R and solar-wind forces. Mars is a less effective trapper because of its small mass, and Venus loses trapping effectiveness primarily because of gravitational disturbances from the Earth.

The question now arises as to whether or not any observational evidence exists for the dust ring that we predict near the orbit of the Earth. There are two sets of observations that are consistent with, but not definitive proof of, a near-Earth dust ring. The first of these is the detection of micrometre- to sub-micrometre-sized meteoroids by the Pioneer 8 and 9 spacecraft. Zook¹⁶ has suggested that these could be created by collisions in a

population of larger parent meteoroids whose spatial density increases with increasing heliocentric distance near 1 AU. McDonnell *et al.*¹⁷, on the other hand, showed that the measurements could also be interpreted on the basis of a spatial density varying with heliocentric longitude. Which interpretation is correct should be resolved with the dust sensor on the Galileo spacecraft scheduled for launch in 1989.

The second set comprise zodiacal-light observations. In an examination of the zodiacal-light data gathered by the Helios spacecraft, Leinert *et al.*¹⁸ failed to find the spatial-density increase suggested by Zook¹⁶. It is possible, however, that a

low-intensity narrow ring near the Earth would not be easily detected by the photometers on Helios, because they did not make observations along the ecliptic plane. On the other hand, it may be significant that the spatial density of particles derived from zodiacal-light studies varies as $r^{-1.3}$ inside a radius of 1 AU (ref. 19) but as $r^{-1.5}$ or higher inverse powers^{20,21} outside of this. Such a result could be caused by a dust ring near the Earth.

We thank Dr E. Everhart for sending us a copy of his numerical integration program and Dr Shin-Yi Su for independently programming and verifying the capture phenomena. We also greatly appreciate a review by Dr J. Burns.

Received 30 August; accepted 20 December 1988.

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Detection of methyl, hydroxymethyl and hydroxyethyl hydroperoxides in air and precipitation

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It is well established that organic peroxides are formed by OH-radical-induced oxidation of hydrocarbons under atmospheric conditions¹. Peroxyacyl nitrates have been known to be constituents of polluted air since the 1950s^{2,3}. In a recent study we have shown that the gas-phase reaction of ozone with a variety of natural and anthropogenic alkenes can contribute to the formation of hydrophilic organic peroxides⁴. Indications that such peroxides are actually present in the environment have been obtained previously by measurements of the peroxide content of cloudwater and rain. In the absence of a specific analytical method the peroxide content after selective enzymatic destruction of the hydrogen peroxide was taken to be the organic peroxide fraction^{5–7}. In this letter we report the determination by high-performance liquid chromatography of methyl (MHP; CH₃OOH), hydroxymethyl (HMP; HOCH₂OOH) and 1-hydroxyethyl (HEP; CH₃CH(OH)OOH)

hydroperoxides, in addition to H₂O₂, and present some preliminary concentration ranges in air and precipitation. The existence of this class of atmospheric trace constituents raises questions about possible adverse biological effects.

Although reversed-phase high-performance liquid chromatography (HPLC) is one of the most important methods of separating polar compounds in aqueous solution, it has been little used in the search for products of tropospheric photo-oxidation. It is superior to gas chromatography in the detection of thermally unstable substances. The method described here (Fig. 1) exploits the stability of hydroxyalkyl hydroperoxides in cold acidic solution by using HPLC to separate them from H₂O₂, which is normally present in much higher concentrations. Directly after separation, both the temperature and the pH of the eluate are increased to convert the hydroxyalkyl hydroperoxides to H₂O₂ and the corresponding aldehydes. H₂O₂ is then detected by the highly specific and sensitive *p*-hydroxyphenyl ethanoic acid/peroxidase fluorescence reaction⁸. Any H₂O₂ initially present in the sample is eluted first from the HPLC column and is detected directly. MHP is also detected directly; it is stable to the higher temperature and pH, but reacts in the presence of horseradish peroxidase.

The peroxides were identified by comparison of their retention times with those of standards (Fig. 2 and Table 1) or by admixture of standards to the samples. The limit of detection is 0.07 µmol l⁻¹, corresponding to 1.4 × 10⁻⁶ µmol per 20 µl of

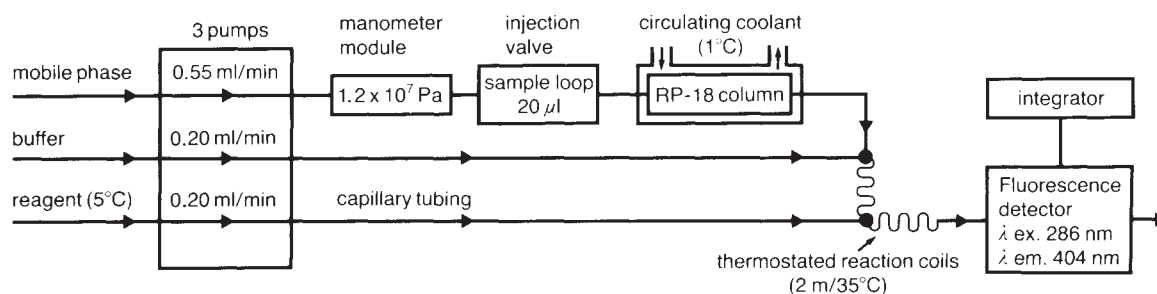


Fig. 1 Analytical system. HPLC: ABIMED, 3 Units Gilson model 302, 5 SC, Manometer module Gilson 802 C. Capillary tubing: o.d. 1/16", i.d. 0.12 mm. Injection valve: Rheodyne 7125. Cryostat: mgw Lauda K2R. Column: Bischoff Type NCI, 4 × 250 mm. Packing: 5-µm Shandon ODS Hypersil. Detector: Hewlett Packard HP 1046 A. Integrator: Hewlett Packard HP 3390. Water: deionized, distilled from KMnO₄, freed from traces of H₂O₂ with catalase, and redistilled. Mobile phase: diluted H₃PO₄, pH 3.5. Buffer: 0.01 M H₂PO₄/NaOH, pH 9.4. Reagent: 0.5 mg *p*-hydroxyphenylethanoic acid (Merck) and 2 mg horseradish peroxidase (EC 1.11.1.7, ~170 U per mg, Merck) in 100 ml 0.01 M KH₂PO₄; 2 mg *p*-hydroxyphenylethanoic acid at peroxide concentrations > 10 µmol l⁻¹. At concentrations > 50 µmol l⁻¹ the sample is diluted.

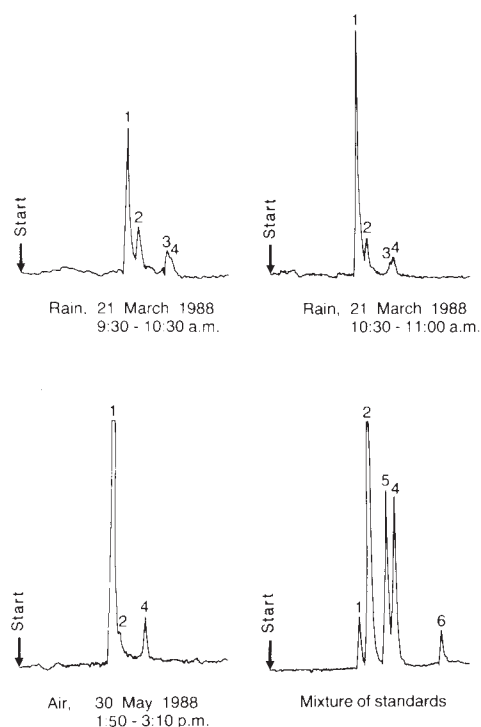


Fig. 2 Typical chromatograms (samples taken at Freising/Munich). See Table 1 for identification of peaks.

sample. Quantification was based on standard solutions of H_2O_2 , MHP and bis(hydroxymethyl)peroxide (BHMP; $\text{HOCH}_2\text{OOCH}_2\text{OH}$) at a concentration of 1 mmol l^{-1} (iodometric titration). Calibration curves for freshly prepared equimolar dilutions of these were identical up to $10 \mu\text{mol l}^{-1}$. HMP and HEP were not available as pure compounds, but always contained small amounts of H_2O_2 and the corresponding bis(hydroxyalkyl)peroxides, so that the calibration was only approximate. The linear range for H_2O_2 can be extended to $50 \mu\text{mol l}^{-1}$ by raising the concentration of *p*-hydroxyphenyl ethanoic acid in the reagent solution, but this is insufficient to extend the linear range for MHP beyond $10 \mu\text{mol l}^{-1}$. Increased reagent also leads to a higher noise level and thereby to a loss in sensitivity. Because of the small difference in retention time ($\Delta R_t = 0.53 \text{ min}$), the detection of HMP is difficult in the presence of much larger amounts of H_2O_2 (ratios of H_2O_2 :HMP > 100:1). HEP and MHP, which have similar concentration ranges in natural samples, are also separated poorly from each other ($\Delta R_t = 0.20 \text{ min}$). The rapid decomposition of HEP at $\text{pH} > 7$ means, however, that the difference between the peak areas measured in acidic and alkaline solution can be used for the quantification of these two peroxides.

During the period from the beginning of February to the end of March 1988, samples of snow from Freising/Munich had an average H_2O_2 concentration of $0.65 \mu\text{mol l}^{-1}$. The highest concentration, $5.5 \mu\text{mol l}^{-1}$, was measured on 24 February in a sample of wet snow taken around noon. In 87 of 93 samples the H_2O_2 content exceeded $0.07 \mu\text{mol l}^{-1}$. HMP could be found in amounts near the detection limit (concentrations $c \approx 0.1 \mu\text{mol l}^{-1}$) in 11 samples.

Rain samples from the same period had significantly higher H_2O_2 concentrations (mean concentration $\bar{c} = 3.5 \mu\text{mol l}^{-1}$; maximum, $c_{\text{max}} = 14 \mu\text{mol l}^{-1}$). In 30 of 75 samples HMP was found at an average concentration of $0.1 \mu\text{mol l}^{-1}$. Higher concentrations of HMP ($c_{\text{max}} = 0.6 \mu\text{mol l}^{-1}$) were more common in those rain samples having $\text{pH} < 5.5$ (compare data for 21 March 1988 in Table 2); HEP ($c_{\text{max}} = 0.3 \mu\text{mol l}^{-1}$), in contrast, was usually found in concentrations near the detection limit and, like MHP ($\bar{c} = 0.1 \mu\text{mol l}^{-1}$; $c_{\text{max}} = 0.3 \mu\text{mol l}^{-1}$), seemed to be nearly independent of pH in the range 4.4–6.1.

The peroxide concentration in 32 rain samples taken in April and May 1988 varied much more widely; for example, only $0.5 \mu\text{mol l}^{-1}$ H_2O_2 was found in water from a heavy shower at 1 p.m. on 20 May, whereas a light drizzle around noon on 17 May contained $110 \mu\text{mol l}^{-1}$. HMP and HEP were not detectable on 16 and 17 May 1988 (Table 2); this can undoubtedly be attributed to the high pH (6.4–7.4) of the samples. In acidic rain ($\text{pH} < 5.5$) the relative concentrations of H_2O_2 , HMP, HEP and MHP were typically 10, 0.7, 0.1 and 0.2 respectively; HMP, HEP and MHP attained maximum concentrations of 1.1, 0.3 and $0.5 \mu\text{mol l}^{-1}$ respectively. BHMP and perethanoic acid, though readily detected by the present method, have not been found in precipitation.

After extended periods of fine weather the first rain, typically with high pH values, contained little H_2O_2 , no HMP or HEP and average amounts of MHP. One or two hours later, however, peak concentrations of H_2O_2 were reached at a pH that was still relatively high. Only as the pH dropped, with prolonged rain, could hydroxyalkyl hydroperoxides be found, along with average amounts of H_2O_2 .

Analyses of 17 aqueous samples obtained by freezing water out of the air at -70°C (Table 3) also attest to the presence of MHP; higher concentrations tend to occur before noon ($c_{\text{max}} = 179 \text{ parts per } 10^{12} \text{ by volume}$). As is to be expected from the higher pH (> 7), HMP and HEP could not be detected in samples taken in the morning. HMP could be found in some samples with $\text{pH} \approx 6$ ($c_{\text{max}} = 15 \text{ parts per } 10^{12} \text{ by volume}$), but only if the analyses were carried out immediately after thawing.

The total peak area of the peroxides observed in particularly acidic rain samples was often less than that of the H_2O_2 peak found after making the sample basic. This indicates that part of the H_2O_2 is initially present in the form of other, as yet undetected, peroxidic compounds. Re-analysis of rain samples that had been stored frozen often gave irregular results (mostly indicating lower peroxide content). Thus, peroxide concentrations should be measured only in fresh samples, and the pH must always be taken into account.

Table 1 Identification of chromatogram peaks

Peak*	Identity	$\bar{R}_t$	Source of standard
1	H_2O_2	5.14 min	Perhydrol (Merck, Darmstadt), 35%
2	HOCH_2OOH (HMP)	5.67 min	For synthesis and properties, see ref. 9.
3	$\text{CH}_3\text{CH}(\text{OH})\text{OOH}$ (HEP)	6.98 min	Synthesis analogous to HMP; $^{13}\text{C-NMR}$ (D_2O /dioxane) $\delta = 21.0$ (q) 101.0 (d).
4	CH_3OOH (MHP)	7.18 min	For synthesis see ref. 10. $^{13}\text{C-NMR}$ (D_2O /dioxane) $\delta = 67.8$ (q); $^1\text{H-NMR}$ ($\text{D}_2\text{O}/\text{CH}_3\text{COOH}$) $\delta = 3.85$ (s).
5	$\text{HOCH}_2\text{OOCH}_2\text{OH}$ (BHMP)	6.69 min	For synthesis and properties, see ref. 11.
6	$\text{CH}_3\text{C}(\text{O})\text{OOH}$	9.63 min	Peroxyethanoic acid (Peroxid-Chemie, München) 40%.

* See Fig. 2.

Table 2 Determination of H₂O₂, methyl, hydroxymethyl and hydroxyethyl hydroperoxides in rain water

			Concentration in $\mu\text{mol l}^{-1}$			
Date/time		pH	H ₂ O ₂	HOCH ₂ OOH	CH ₃ CH(OH)OOH	CH ₃ OOH
21 March 1988*	8:00–9:00 a.m.	4.7	5.3	0.3	0.1	0.2
	9:30–10:30 a.m.	4.8	2.3	0.4	0.3	0.2
	10:30–11:00 a.m.	5.4	5.8	0.6	0.2	0.3
	12:15 p.m.‡	6.0	8.6	0.2	ND	0.1
	12:30 p.m.	5.9	7.0	0.2	0.1	0.2
	1:00 p.m.	6.1	5.8	0.2	0.1	0.1
	1:30 p.m.	5.9	5.3	0.4	0.1	0.1
	2:15 p.m.	5.1	6.3	0.5	0.1	0.1
	3:00 p.m.	4.7	6.0	0.6	0.1	0.1
	3:15 p.m.	4.8	6.0	0.5	0.1	0.2
	3:30 p.m.	4.4	6.8	0.6	0.1	0.2
	4:00 p.m.	4.4	6.7	0.5	0.1	0.1
	4:15 p.m.	7.3	9.0	ND	ND	0.2
	4:45 p.m.	7.4	9.3	ND	ND	0.1
	5:00 p.m.	7.0	35.2	ND	ND	0.3
16 May 1988†	5:30 p.m.	6.7	63.1	ND	ND	0.3
	10:15 a.m.	7.1	20.0	ND	ND	0.3
	11:25 a.m.	7.0	57.4	ND	ND	0.3
	12:30 p.m.	6.4	110.6	ND	ND	0.4
17 May 1988†	1:30 p.m.	6.4	28.8	ND	ND	0.1
	1:30 p.m.	4.4	36.2	>0.8§	0.3	0.5
	1:45 p.m.	4.2	57.1	>0.8§	0.3	0.5
19 May 1988*	3:25 p.m.	4.1	32.4	>0.8§	0.3	0.3

Rain at Freising/Munich was caught by a glass funnel (30 cm diameter) during the reported time period or until at least 0.5 ml was collected. The samples were analysed immediately. ND, not detectable.

* Sampling during an extended rain period.

† Sampling at the beginning of a rain period.

‡ Shoulder of the H₂O₂ peak, not precisely quantified.

Table 3 Determination of H₂O₂, methyl and hydroxymethyl hydroperoxides in air

		Air temperature (°C)	Relative humidity (%)	Sample weight (g)	Sample pH	Concentration (parts per 10 ¹² by volume)		
Date/time						H ₂ O ₂	HOCH ₂ OOH	CH ₃ OOH
30 May 1988	9:50–11:10 a.m.	15	61	3.8	7.4	453	ND	179
	12:05–1:25 p.m.	17	61	3.9	6.1	1,471	4*	73
	1:50–3:10 p.m.	18	59	3.9	6.1	914	4*	77
	3:30–4:50 p.m.	17	60	4.0	6.0	455	15*	40

0.5 m³ air at Freising/Munich (1.2 m above ground) was drawn at 6.25 l min⁻¹ with a gas sampler (Desaga GS 312) through an efficient cold trap (Brand) at -70 °C; the frozen sample was gently thawed and analysed immediately. ND, not detectable.

* Shoulder of the H₂O₂ peak, not precisely quantified.

In general, peroxide concentrations increased significantly whenever snow turned into rain. It is possible that snowflakes are less capable than raindrops of taking up peroxides while falling or that decomposition reactions occur on the ice crystals, as was observed in the analysis of frozen rain samples. The latter effect might reduce the peroxide content of samples frozen out of air. The analysis of these samples is further complicated by the possibility that some peroxides are not formed in significant amounts until the precursors are concentrated in the cold trap.

The range and the variation with time of the H₂O₂ concentrations in the present study are comparable with those reported by others^{5,7,12–14}. In addition, the sum of the organic peroxide concentrations, on average ~1–10% of the H₂O₂ concentration, is in agreement with the values of Kelly *et al.*⁵ and Lazrus *et al.*⁷. The occasional exceptionally high values of more than 30% organic peroxide in refs 5 and 7 were also found in very acidic rain samples. The absence of BHMP (the product of HMP and CH₂O in acidic solution) can be attributed to the low ratio of CH₂O to H₂O₂ in rain water. Perethanoic acid, on the other

hand, which has been postulated as an intermediate in the atmospheric degradation of hydrocarbons^{1,15}, is too rapidly hydrolysed to H₂O₂ and ethanoic acid in dilute aqueous solution to be detected in rain samples by the present method. MHP has been found in simulated atmospheric oxidation of hydrocarbons such as methane and is predicted to be a trace constituent of the troposphere^{16–18}. Its low concentration in the air on sunny afternoons and its occurrence in many of our rain samples appear to confirm that photolysis and washing out by rain are major sinks for this peroxide¹⁹.

There are two plausible atmospheric pathways for the formation of hydroxyalkyl hydroperoxides. The main route may be the addition of H₂O₂ to aldehydes under acidic conditions. Of importance in this context is the fact that H₂O₂ creates its own acidic environment by oxidizing the ubiquitous atmospheric SO₂ to sulphate^{20,21}. The alternative route to these peroxides involves the addition of water to the so-called Criegee intermediate, R₁R₂COO, of the reaction of ozone and alkenes²² (R₁ and R₂ are alkyl groups or hydrogen); no acid is required for this addition, but the lifetime of the peroxides is of course far longer

if acid is present. The formation of hydroxyalkyl hydroperoxides was established in our earlier simulations of gas-phase ozonolysis of naturally occurring alkenes (for example, terpenes, isoprene and ethene)⁴. In a recent study¹³, concentrations of H₂O₂ were sometimes found to be much higher in a forest than those just outside it, suggesting that some of the H₂O₂ may be formed by decomposition of hydroxyalkyl hydroperoxides produced in the rapid reaction of ozone with biogenic alkenes.

There are too few studies of the effects of the peroxides described here on intact organisms to justify any definite statements about their probable environmental significance. Various physiological effects of HMP have, however, been examined in detail^{23,24}. Among these is the irreversible inhibition of horseradish peroxidase. Inhibition of plant peroxidases by low concentrations of HMP might greatly increase the toxic effects of H₂O₂, which have been found in experiments on trees²⁵. Damage of a type attributable to peroxides has often been mentioned in descriptions of *Waldsterben* (forest death)²⁶, but simulations using environmentally relevant concentrations of ozone and peroxyacetyl nitrate, the usual representative photo-oxidants, have failed to reproduce all the symptoms of this damage^{27,28}. These studies may have taken too little account of the possibility that the products of ozone, not ozone itself, are responsible for the damage to plants. If during the experiment the reaction products are not allowed to build up in the air surrounding the plants, as they probably do in nature, fumigation with ozone may not produce the effects observed in forests. A recent set of experiments on pea seedlings in a laboratory showed that ozone causes more damage when in combination with stress ethylene²⁹, a result which is consistent with our suggestion. Studies are urgently needed to determine to what extent and by what route terpene oxidation products, among them the peroxides discussed here, can penetrate plant tissues and what their effects on these tissues are. This work indicates that damage by hydroxyalkyl hydroperoxides should be greater in acidic environments. It is perhaps relevant that there are several reports that ozone and acidity act synergistically in damaging plants^{30,31}.

Received 30 September; accepted 7 December 1988.

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Three-dimensional micromagnetic modelling of ferromagnetic domain structure

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The fidelity of magnetic recording materials (whether they be synthetic crystals on a recording tape, or natural crystals in rocks recording changes in the Earth's magnetic field) depends on the uniformity of their magnetic structure. Such structure is determined by minimizing the energies arising from atomic magnetic dipole interactions. Previous micromagnetic models of coupled spin structures have contained well defined constraints to make the calculations tractable. By using a supercomputer, we have been able to perform unconstrained calculations of minimum energy structures for cubic assemblies of up to $22 \times 22 \times 22$ exchange-coupled spins. The critical size for transformation from a uniform single-domain structure to a non-uniform three-dimensional structure is about $0.1 \mu\text{m}$ for magnetite, as found previously from one-dimensional modelling. However, a variety of different non-uniform structures are possible, with energies and magnetic moments much less than those of conventional lamellar domains. The predicted moments of unweighted combinations of these states agree well with experimental measurements on magnetite in the size range 0.08 – $0.5 \mu\text{m}$. Surface spin structures are such as to minimize flux leakage out of the particle and might be misleadingly imaged by the Bitter colloid technique as indicating a single-domain state.

Early micromagnetic models of domains were analytic^{1,2}, and permitted only a one-dimensional variation in the spin angle between domains of uniform magnetization³. Computer techniques have facilitated numerical models that allow non-uniform solutions^{4,5}. Although initially such models were one-dimensional, LaBonte⁶ and more recently Aharoni and Jakubovics⁷, Fredkin and Koehler²⁶, and Zhu and Bertram²⁷ have achieved two-dimensional solutions.

One- and two-dimensional solutions have proved useful in investigating the magnetic stability of ferromagnetic crystals^{8–10}, but there is no doubt that the true magnetization structure is three-dimensional. Here we describe a fully three-dimensional micromagnetic model, in which no constraints are placed on the direction of magnetization and the bodies considered are finite in all three dimensions.

For simplicity, we consider cubes of magnetite in zero external field at room temperature. Generalization to other temperatures, fields, crystal forms and phases is straightforward and will be described elsewhere. Each cube is divided into N^3 sub-cubes. The magnetic moment of any sub-cube is the average over a very large number of atomic spins and is represented by a dipole $\mathbf{m}_i$ at the centre of the sub-cube. All dipoles have the same magnitude m_i but their directions vary.

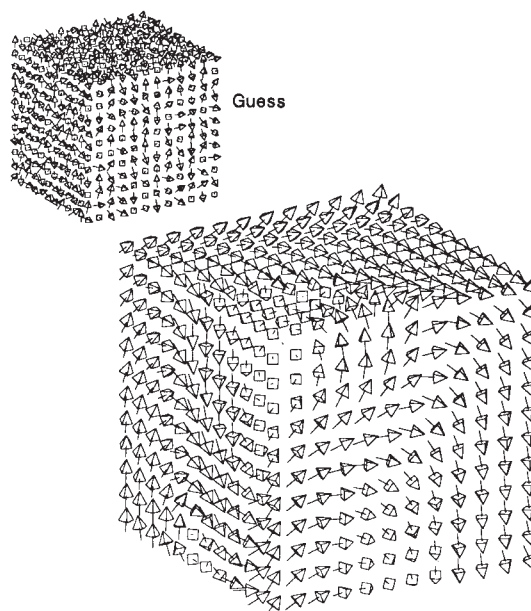
The contributions to the total magnetic energy of a cube were formulated as described by Brown¹¹. (1) The exchange energy, E_E , is due to coupling of the spins $\mathbf{S}_i$ and $\mathbf{S}_j$ of nearest-neighbour atoms:

$$E_E = -2 \sum_x \sum_y \sum_z J_E \mathbf{S}_i \cdot \mathbf{S}_j$$

where J_E is the exchange integral. As in other micromagnetic calculations¹², the J_{AB} (inter-sublattice) interaction is assumed to dominate. We also assume linear variations of the atomic spin directions in the x , y and z directions between the centres of the sub-cubes. (2) Magnetocrystalline anisotropy energy, E_A , is due to spin-lattice coupling:

$$E_A = \sum_x \sum_y \sum_z (K_1(\alpha_1^2 \alpha_2^2 + \alpha_2^2 \alpha_3^2 + \alpha_3^2 \alpha_1^2) + K_2 \alpha_1^2 \alpha_2^2 \alpha_3^2)$$

Fig. 1 A minimum-energy state of 12^3 exchange-coupled dipoles, obtained by an unconstrained minimization routine, starting from the initially guessed structure shown in the insert. The minimum-energy state involves no imposed symmetry from a random initial guess. Grain size = $0.2 \mu\text{m}$ cube. Reduced energy = 0.54. $M_{\text{rs}}/M_s = 0.28$.



where K_1 , K_2 are the anisotropy constants and α_1 , α_2 , α_3 are the direction cosines of $\mathbf{m}_i$. (3) Magnetostrictive strain energy, E_s , is due to lattice deformation caused by the magnetization of the crystal:

$$E_s = \frac{3}{2} \sum_x \sum_y \sum_z \lambda \sigma \cos^2 \phi$$

where λ is the magnetostriction constant, σ is the magnetoelastic stress and ϕ is the angle between the stress and magnetization direction. (4) Demagnetizing energy, E_D , is due to magnetostatic dipole-dipole interaction:

$$E_D = -\frac{1}{2} \sum_x \sum_y \sum_z \mathbf{m}_i \cdot \mathbf{H}_i$$

where $\mathbf{H}_i$ is the magnetic field at the location of $\mathbf{m}_i$ that results from all the other dipoles.

Stable magnetic structures were determined by finding minima of the sum $E_E + E_A + E_D$, using previously published values for the saturation magnetization¹³, the anisotropy constant¹⁴ and the exchange constant¹⁵. The magnetostrictive energy in magnetite is nearly two orders of magnitude smaller than any of the other energies involved and was not considered further in this initial study. The anisotropy of the solutions shown in Fig. 2 was simplified to a uniaxial anisotropy along the $+z$ direction. Solutions with cubic anisotropy, such as those in Fig. 1, were not significantly different, because of the dominance of magnetostatic energy which strongly favours alignment of spins parallel to crystal surfaces.

The localized energies E_E and E_A are easy to compute. E_D , however, is long-ranged and nonlinear. Even for a relatively low resolution of 12^3 sub-cubes, the demagnetizing energy requires ~ 1.5 million interaction calculations per iteration. The computation can be reduced considerably by rewriting the demagnetizing energy in the manner of Rhodes and Rowlands¹⁶, as the product of an invariant demagnetizing coefficient and variable equivalent magnetic charges on the surfaces of the sub-cubes¹⁰. In one-dimensional models, only interaction coefficients between parallel sheets of magnetic charge are needed. For the three-dimensional problem, the interaction coefficients between orthogonal sheets must also be calculated.

The minimization was performed using the conjugate-gradient method on a Cray X-MP/22 supercomputer. Second-order methods converge faster but memory constraints did not allow storage of the hessian matrix. Figure 1 demonstrates the effectiveness of the minimization technique. This minimum-

energy state was obtained in just under 50 minutes of computer time from a random initial guess.

Because of the large number of dipoles, numerous different minimum-energy states exist. To make the computations more efficient and allow us to increase the resolution to 22^3 sub-cubes, we next restricted our attention to symmetric solutions in which the structure in a given octant of the cube is either symmetric or antisymmetric with respect to that of a reference octant. Non-symmetric minimum-energy states (such as that in Fig. 1) always had higher energies than corresponding symmetric states with similar features (for example, Fig. 2d). All our solutions were tested for stability by removing the symmetry requirement and solving for a new minimum-energy structure.

Symmetric structures of various types are shown in Fig. 2. There are no lamellar domains of the classic Kittel² type. Regions of uniform magnetization correspond to areas of constant colours. These domains have dipoles aligned in the $\pm z$ directions. The net moments of the cubes are also aligned in this direction. In the single-domain (or type 1A) solution (Fig. 2a) the moments are not all exactly parallel, as in a classical single-domain grain; spins are deflected at the corners, where the demagnetizing field is strongest¹⁷. As the grain size increases the deflections at the corners become more pronounced. Above $\sim 0.2 \mu\text{m}$ a new type of structure arises, in which the moments at the cube edges reverse to form domains aligned in the $-z$ direction (Fig. 2b). This solution (type 1B) is a five-domain grain, having four domains in the $-z$ direction at the cube edges and a core domain in the $+z$ direction.

A classical two-domain grain (type 2 solution) can also be generated (Fig. 2c) which has a lower energy than the single-domain or five-domain solutions in the grain size range 0.096 – $0.35 \mu\text{m}$. Thus the critical single-domain size d_0 in magnetite is $\sim 0.1 \mu\text{m}$, as predicted by one-dimensional theories^{8,10,15,18}, and the equilibrium structure above d_0 is a two-domain state with large closure domains and zero net moment. The solution (type 3) that is energetically the most favourable above $\sim 0.35 \mu\text{m}$ is shown in Fig. 2d; this also a five-domain grain but is characterized by curling of the magnetization throughout the body of the grain.

The energies of these structures are between 2 and 3 times lower than those of one-dimensional solutions in the same grain-size range. This reduction in energy is due to the fact that the spins are free to rotate and thus to reduce the surface magnetic poles. A similar reduction of the surface-pole density was noted by LaBonte⁶ for two-dimensional solutions of 180° domain walls in thin films. The net effect is that spins near the

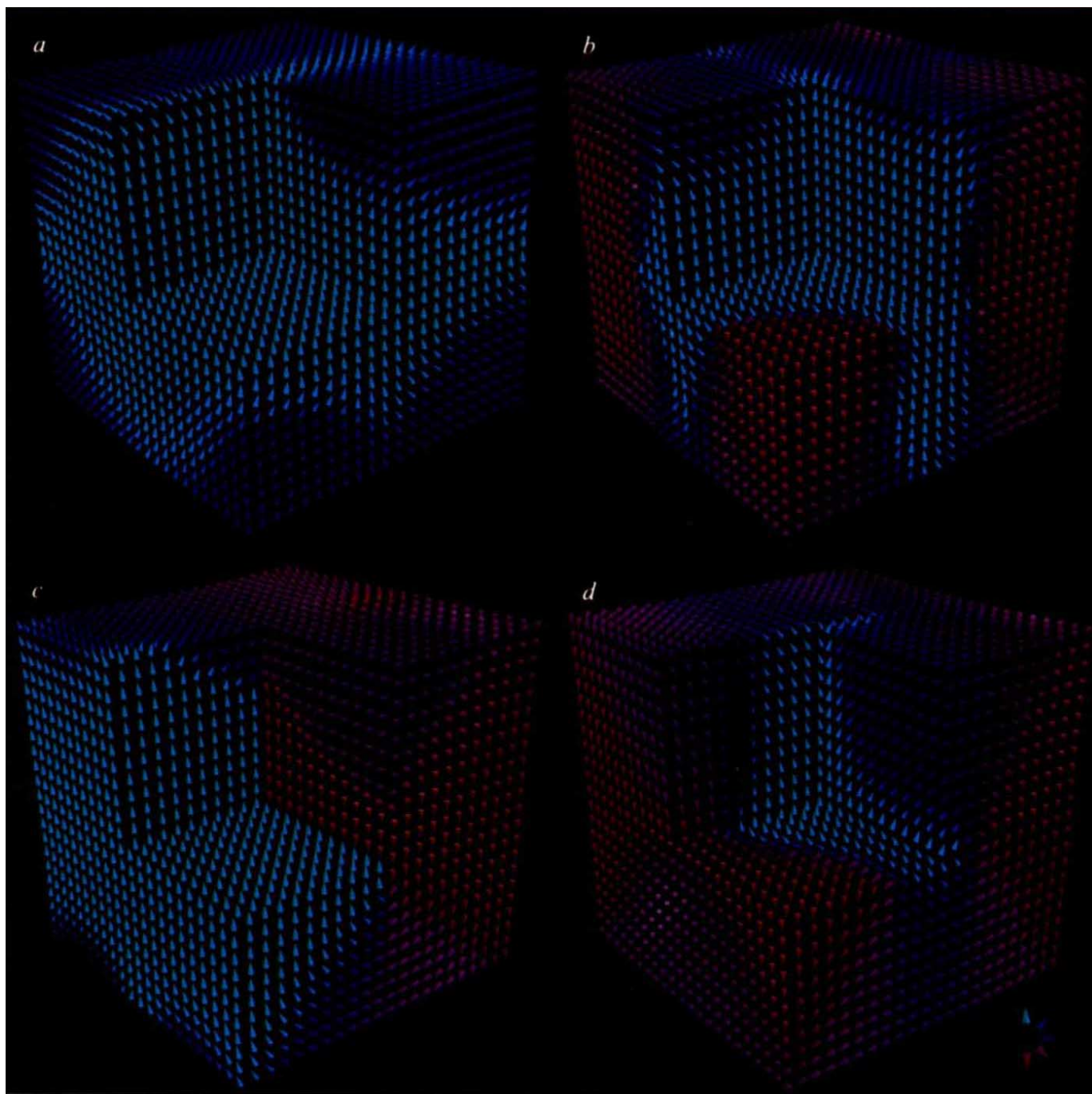


Fig. 2 Various types of symmetric solutions for $0.2\text{ }\mu\text{m}$ cube grain size. The solid cones representing the magnetic dipoles are coloured according to their angle from the vertical: light blue represents up and dark red represents down. *a*, A single-domain structure (type 1A), showing deflection of the magnetization at the cube corners. Reduced energy = 0.81; $M_{rs}/M_s = 0.89$. *b*, A five-domain structure (type 1B), obtained using the same symmetry as that of type 1A. Reduced energy = 0.78; $M_{rs}/M_s = 0.32$. *c*, A two-domain structure (type 2), with large closure domains. Reduced energy = 0.39; $M_{rs}/M_s = 0.0$. *d*, A five-domain structure (type 3) dominated by internal flux loops. Reduced energy = 0.50; $M_{rs}/M_s = 0.12$.

grain surface tend to lie parallel to the surface, so that Néel walls are favoured over Bloch walls, in which spins rotate in a plane perpendicular to the surface. Where Bloch walls do occur, as on the side faces of Fig. 2*b*, they are much narrower than predicted by one-dimensional models. Therefore Bloch walls themselves will not contribute significantly to the net magnetic moment of the grain.

We can compare our predictions of magnetic moments and Bitter patterns with experimental observations. Saturation remanence (M_{rs}) is a bulk magnetic parameter which has been measured for well-sized dispersed magnetite grains. It is likely

that all the different domain configurations that are possible in a grain of a particular size will coexist and contribute to M_{rs} . Assuming equipartition of the permitted symmetric states, we predict the grain-size dependence of the reduced saturation remanence M_{rs}/M_s plotted in Fig. 3. A factor of 0.5 has been incorporated in the theoretical curve to allow for random orientations. This value is appropriate for crystals dominated by uniaxial shape anisotropy, as has shown to be the case^{19,20} for many of the samples whose data appear in Fig. 3. There is good agreement with the published experimental data over the range 0.08–0.5 μm .

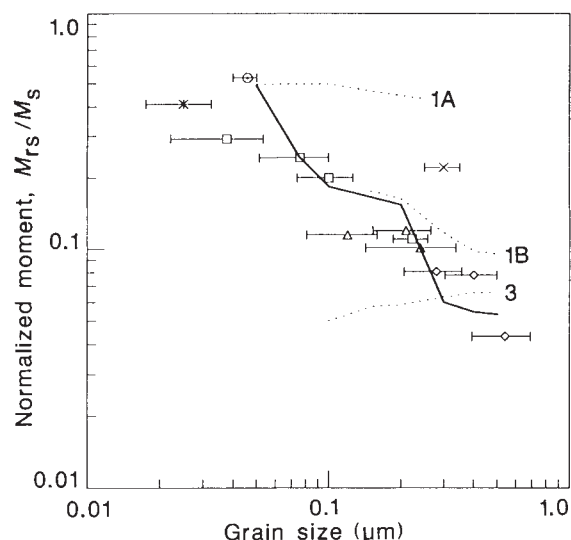


Fig. 3 Predicted grain-size dependence of reduced saturation remanence M_{rs}/M_s (solid curve) compared with experimental data. The dotted curves are the theoretical predicted values for the different structures shown in Fig. 2. Note that the two-domain structure (type 2) has zero net moment. The experimental data were obtained from: Δ Levi and Merrill²⁰, $\square$ Dunlop²¹, * Özdemir and Banerjee²², $\times$ Schmidbauer and Veitch²³, $\circ$ Moskowitz *et al.*²⁴ and $\diamond$ Dunlop and Argyle²⁵.

Bitter colloid patterns form in response to the magnetic field gradient normal to the surface of a grain. Because Néel walls do not generate large normal field gradients, even quite complex structures, such as that of Fig. 2d, might not be imaged by the Bitter technique, so that such a grain might be interpreted as comprising a single domain.

The results obtained so far show good agreement with bulk measurements of M_{rs} . Unfortunately we lack the experimental observations of Bitter patterns on submicrometre grains that are needed to test our predicted structures more rigorously. However, testable predictions of temperature and field dependencies of these structures are currently being investigated.

We thank the Ontario Centre for Large Scale Computation, particularly Dr Nina Bregman, James Rowell and Anna Pezacki for their help. We also thank Randy Enkin, whose work on one-dimensional modelling stimulated this study, and Shiu-Hong Lui for his efforts in coding much of the computer program. This work was supported by an operating grant from NSERC of Canada.

Received 6 September; accepted 20 December 1988.

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Sensitivity of climate and atmospheric CO₂ to deep-ocean and shallow-ocean carbonate burial

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Previous models of physical and biogeochemical controls of the evolution of atmospheric CO₂ and climate over hundreds of millions of years have neglected the effect of variations in the balance between shallow-ocean and deep-ocean carbonate deposition. This is important because the relative proportions of carbonate burial in the two reservoirs have changed over time. Here I set up a model of the carbonate-silicate geochemical cycle that distinguishes carbonate masses produced by the two types of burial and shows that reasonable increases in deep-ocean burial could produce substantial warmings over a few hundred million years. The model includes exchanges between crust and mantle; transients from burial shifts are found to be sensitive to the fraction of non-degassed carbonates subducted into the mantle. Without the habitation of the open ocean by plankton such as foraminifera and coccolithophores, today's climate would be substantially colder.

Over geological timescales, atmospheric CO₂ (P_{atm}), and therefore global temperature (T), is controlled by differing balances of sources and sinks¹⁻⁷. Models have examined, in varying degrees of detail, forcings upon and changes in these source and sink terms, including net organic-carbon burial³, sea-floor generation rate and continental area^{2,3}, P_{atm} and T feedbacks in the weathering sink^{1,2,5} (the chemical weathering of continental silicates, followed by carbonate burial), and the effect of the terrestrial biosphere^{5,6}. Until now, models of the geochemical carbonate-silicate cycle that explicitly calculated P_{atm} have failed to resolve the different dynamics of pelagic and continental carbonates. The relative proportions of carbonate entering these two reservoirs have changed over time^{8,9}, and there is strong evidence that the proportion of pelagic burial has increased substantially since the middle Jurassic⁹⁻¹¹.

A fundamental division of carbonates into those deposited in shallow water and those deposited in deep water is required to examine how their different dynamics could affect atmospheric CO₂, primarily because, mass for mass, the metamorphic flux of CO₂ to the atmosphere is much larger from pelagic than from continental carbonates. Burial in shallow water produces carbonates that will eventually be weathered or degassed on the continents; these continental carbonates decay statistically at a rate that is slower by an order of magnitude than that of the pelagic carbonates⁹. Burial in deep water produces pelagic carbonates whose sink consists of the subducting slabs at plate boundaries¹². There, under conditions of higher heat and pressure, they may either be metamorphosed to silicates, accompanied by outgassing of CO₂ which eventually enters the atmosphere, or remain as carbonates on the slabs as they are subducted into the mantle¹³. The cycle of carbon between the mantle and crust has been shown to be important in the geochemical carbon cycle¹²⁻¹⁴, but has not yet been incorporated explicitly into models for P_{atm} .

Our model for P_{atm} and masses of continental (M_c) and pelagic (M_p) carbonates is illustrated in Fig. 1, with interconnecting fluxes of carbon, F . Because the organic-carbon cycle is of only secondary importance³, the only sink for P_{atm} considered here is that associated with silicate weathering (F_{sw}). The weathering fluxes— F_{sw} and that from the continental carbonates, F_{cw} —are derived from their present-day values ($F_{sw,ref}$ and $F_{cw,ref}$) using a non-dimensional weathering-rate factor $f_{wr}(T)$ (ref. 2). The temperature dependence of f_{wr} (equation 2) incorporates effects directly related to T as well as the effect of

temperature on global runoff; other forms for weathering are available^{1,5}, but would not essentially alter the conclusions. As in ref. 2, the weathering formulations assume that silicate weathering is unlimited, but that M_c affects carbonate weathering (see equations 1a, b). A greenhouse equation² relates P_{atm} and T (equation 3). Metamorphic release of CO_2 from continental carbonates is assumed to be proportional to M_c , and for simplicity, the flux of pelagic carbonates entering the subduction zones is also assumed to be proportional to M_p , as in ref. 9 (equations 4a, b). In reality, there is a varying delay during spreading between carbonate deposition above the carbonate compensation depth on the ridges and its subduction several tens of millions of years later. This delay is neglected here as we are concerned only with the fundamental dynamics of the system. Therefore, our equations are

$$F_{\text{sw}} = F_{\text{sw,ref}} f_{\text{wr}} \quad ; \quad F_{\text{cw}} = F_{\text{cw,ref}} f_{\text{wr}} \frac{M_c}{M_{c,\text{ref}}} \quad (1a, b)$$

$$f_{\text{wr}} = 1.0 + 8.7 \times 10^{-2} (T - T_{\text{ref}}) + 1.9 \times 10^{-3} (T - T_{\text{ref}})^2 \quad (2)$$

$$T - T_{\text{ref}} = 2.88 \ln \left(\frac{P_{\text{atm}}}{P_{\text{atm,ref}}} \right) \quad (3)$$

$$F_{\text{cm}} = k_{\text{cm}} M_c \quad ; \quad F_{\text{sub}} = k_{\text{sub}} M_p \quad (4a, b)$$

We introduce a burial parameter β ($0 \leq \beta \leq 1$), representing the way in which physical and biogeochemical ocean processes proportionate the weathering fluxes between the pelagic carbonates, $\beta(F_{\text{sw}} + F_{\text{cw}})$, and the continental carbonates, $(1 - \beta)(F_{\text{sw}} + F_{\text{cw}})$. Abiotic carbonate precipitation in the hydrothermal cooling systems of mid-ocean ridges¹⁵ could also be considered to contribute to β . The parameter β is obviously the same for both silicate and carbonate weathering, because precipitation does not distinguish calcium ions derived from different rock types.

The subduction flux, F_{sub} , is divided, using a subduction parameter σ ($0 \leq \sigma \leq 1$), into a flux entering the mantle, σF_{sub} , and a flux representing metamorphic decarbonation $(1 - \sigma)F_{\text{sub}}$, which produces a source flux of CO_2 to the atmosphere. Transfer from M_p to M_c during lateral accretion of pelagic sediments to a continent at a convergent plate boundary is assumed to be negligible in this context. Finally, the flux of outgassed CO_2 from the mantle entering the atmosphere from the ocean ridges, F_r , is assumed to be constant. The governing equations for the three reservoirs are then:

$$\text{for } M_c: \quad \frac{dM_c}{dt} = (1 - \beta)F_{\text{sw}} - \beta F_{\text{cw}} - F_{\text{cm}} \quad (5)$$

$$\text{for } M_p: \quad \frac{dM_p}{dt} = \beta(F_{\text{sw}} + F_{\text{cw}}) - F_{\text{sub}} \quad (6)$$

$$\text{for } P_{\text{atm}}: \quad F_r + F_{\text{cm}} + (1 - \sigma)F_{\text{sub}} - F_{\text{sw}} = 0 \quad (7)$$

which control M_c , M_p and P_{atm} respectively. A steady-state balance for P_{atm} must be maintained in the ocean/atmosphere system at all times over the timescales in this problem^{1,2,5,7}, even though P_{atm} varies with time in the longer term.

Figure 2 shows the results of perturbing an initial steady-state. The initial values are broadly consistent with the relationships in refs 2, 9, and 13 (see figure caption). This state is supposed to represent neither the present, although it draws upon many of the relationships that exist therein, nor any particular point in the past. Rather, it serves as a generic condition from which to study the response-dynamics of the system.

We introduce a step increase to β , $\Delta\beta$, at time $t = 0$. In Fig. 2, a trio of runs with $\Delta\beta = 0.1, 0.3$ and 0.5 shows that global warming follows the step increase to a degree that increases with larger $\Delta\beta$. For all of these cases, which have $\sigma = 0.5$ (a possible upper bound¹³), T is nearly maximal between 100 and 200 Myr after the β -step. The peak warmings for the three cases,

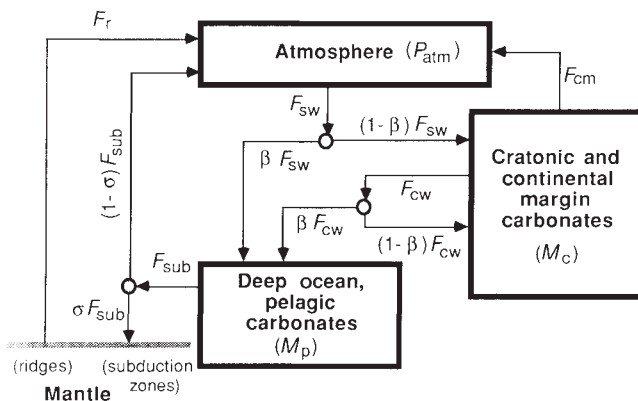


Fig. 1 Flows of carbon between reservoirs. The weathering fluxes associated with the weathering of silicates (F_{sw}) and continental carbonates (F_{cw}) are diverted by a burial parameter (β), which represents the degree to which physical and biological ocean processes deposit the carbon as carbonates either in shallow water, to become continental carbonates, or in deep water, as pelagic carbonates. The subduction flux (F_{sub}) either enters the mantle as carbonate or is metamorphosed, accompanied by CO_2 degassing into the atmosphere; this division is quantified with a subduction parameter (σ). The model also incorporates CO_2 fluxes to the atmosphere from the ocean ridges (F_r) and from continental metamorphism (F_{cm}).

respectively, are 2, 6 and 11 °C, and are followed by much slower cooling. These two trends—warming followed by cooling—derive from different rates of equilibration between sub-systems. In general, following the β -step, the increased burial flux to the deep ocean increases the pelagic carbonate mass, which increases CO_2 input to the atmosphere. Because of their relatively large mass, a decrease of only a few per cent in the mass of the continental carbonates causes a proportionately much larger increase to the pelagics. Over longer times, adjustment occurs between the total crustal carbonate system and the mantle, as the masses of both continental and pelagic carbonates decrease. In Fig. 2, as the pelagic mass declines, so does the metamorphic source flux of CO_2 to the atmosphere, hence giving rise to cooling. Note that the system is out of equilibrium for the Phanerozoic-length period of time shown in Fig. 2.

Because the present-day subduction efficiency σ is not well constrained (lying between 0 and 0.5 (ref. 13)), we have also examined the effect of varying this parameter. Using one case from the first trio of runs as a reference ($\Delta\beta = 0.1$, $\sigma = 0.5$), σ is decreased to 0.3 and 0.1. For a fixed $\Delta\beta$, a smaller σ gives a larger rate of warming, a larger peak ΔT and a longer time to reach the peak ΔT . For example, ΔT rises to ~8 °C by 400–500 Myr after the β -step, for $\sigma = 0.1$. This sensitivity arises because a smaller σ means that a higher metamorphic source flux of CO_2 to the atmosphere from the subduction zones is maintained for a given mass of pelagic carbonate. Figure 2 shows that substantial climatic changes follow even small shifts in β if σ is small, and also occur with larger values of σ if the shifts in β are larger.

The main objective of this study is to point out that partitioning of ocean carbonate burial must be incorporated in studies of the evolution of atmospheric CO_2 and climate. Evolutionary changes, such as the proliferation of coccolithophores after the Triassic and of globigerinacean foraminifera after the late Jurassic, shifted a significant portion of global carbonate deposition from the shelves to the deep ocean⁸. Wilkinson and Walker⁹ propose that a gradual shift in β from about 0 to 0.6 has occurred over the past 140 Myr. (β might never have been zero, for hydrothermally induced carbonate precipitation at ocean ridges¹⁵ may prevent β from falling below a certain minimum value. Also, even in the absence of CaCO_3 -precipitating organisms in the open ocean, abiotic deep-ocean precipitation would have been required to maintain chemical balance if sea level

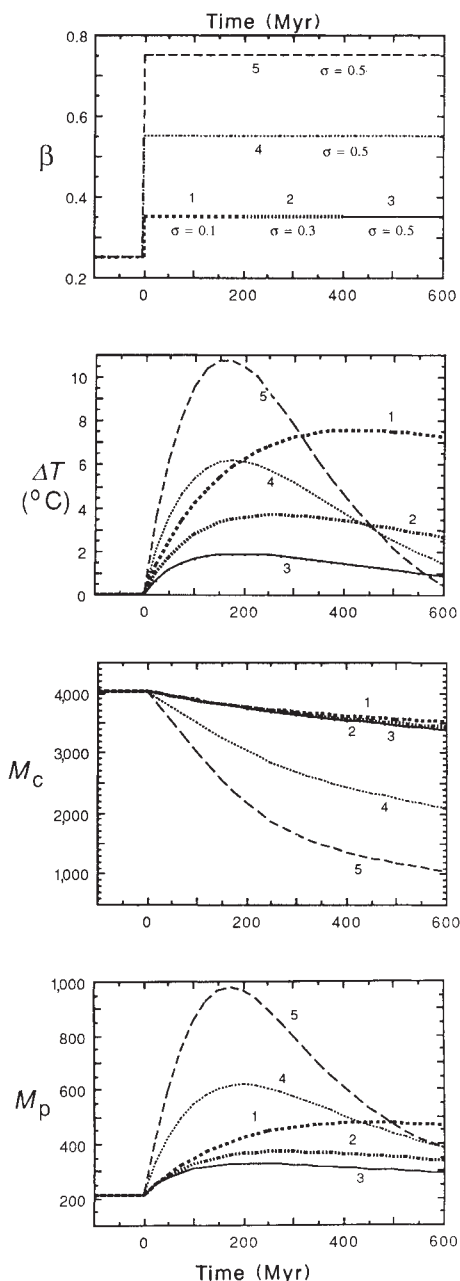


Fig. 2 Results of model runs, demonstrating the effect of a step increase, $\Delta\beta$, in the burial parameter β , for $\Delta\beta = 0.1, 0.3$ and 0.5 (cases 5, 4 and 3, with the subduction parameter $\sigma = 0.5$). To demonstrate sensitivity to σ , cases 3, 2 and 1 have $\sigma = 0.5, 0.3$ and 0.1 , respectively, each with $\Delta\beta = 0.1$. All cases have identical initial values for β, T, M_c and M_p , and are initially in steady-state. These identical initial values can be set for different values of σ by varying F_r to satisfy $F_r = \sigma F_{\text{sub}}$, with F_{sub} taking the value for a steady-state solution with $\sigma = 0$. To satisfy the equations described in the text, the system requires reference values. We use those in ref. 2: $F_{\text{sw,ref}} = 5.9 \times 10^{18} \text{ mol myr}^{-1}$; $F_{\text{cw,ref}} = 12.5 \times 10^{18} \text{ mol myr}^{-1}$; $M_{\text{c,ref}} = 5,000 \times 10^{18} \text{ mol}$; $T_{\text{ref}} = 15^\circ\text{C}$; $P_{\text{atm,ref}} = 310 \mu\text{atm}$. Following ref. 9, $k_{\text{sub}} = 0.022 \text{ myr}^{-1}$ (approximately the inverse of the average age of ocean floor¹⁰). The value for k_{cm} is chosen such that $k_{\text{cm}} \ll k_{\text{sub}}$ and k_{cm} is less than the weathering rate constant of continental carbonates ($\sim 0.002 \text{ myr}^{-1}$ in ref. 9) but is not negligible; we use $k_{\text{cm}} = 0.0005 \text{ myr}^{-1}$. Somewhat arbitrarily setting $\beta = 0.25$ for the initial steady-state gives a reasonable crust and mantle silicate-carbonate cycle for perturbation studies, specifically, initial conditions of $M_c = 4,020 \times 10^{18} \text{ mol}$; $M_p = 210 \times 10^{18} \text{ mol}$ (for present-day values¹⁰ $M_p < 0.1 M_c$); $P_{\text{atm}} = 380 \mu\text{atm}$; $T = 15.6^\circ\text{C}$; $F_{\text{cm}} = 2.0 \times 10^{18} \text{ mol myr}^{-1}$; $F_{\text{cw}} = 10.6 \times 10^{18} \text{ mol myr}^{-1}$; $F_{\text{sw}} = 6.2 \times 10^{18} \text{ mol myr}^{-1}$; $F_{\text{sub}} = 4.1 \times 10^{18} \text{ mol myr}^{-1}$. The temperature is scaled such that $\Delta T = T - 15.6^\circ\text{C}$. For $\sigma = 0.5, 0.3$ and 0.1 , respectively, $F_r = 2.1, 1.3$ and $0.4 \times 10^{18} \text{ mol myr}^{-1}$. These values for F_r are within the lower end of the range estimated by ref. 13, and are reasonable in view of the fact that a steady-state atmosphere/ocean system requires $F_r < F_{\text{sw}}$. The system uses a fourth-order Runge-Kutta scheme²⁰ with time steps of 1 Myr; here time is scaled such that the step increase in β occurs at $t = 0$.

was below the shelf.) Wilkinson and Walker attributed this shift to ecological changes, as have others, but also made a strong case for the alternative of linking β directly to sea level as a result of changes in the area of shallow epicontinental seas. Because β could decrease as sea level rises and vice versa, falling sea level during the past 100 Myr could explain an increase in β . But regardless of the cause, such an increase in β over the past 100 Myr is widely accepted⁹⁻¹¹. According to Fig. 2, shifts in β of this magnitude should now be affecting the Earth's carbonate-silicate cycle, atmospheric CO_2 and climate.

The value of the subduction parameter σ is highly uncertain. Within our model, the peak of 11°C in the time history of ΔT with $\Delta\beta = 0.5$ and $\sigma = 0.5$ is moderated to $\sim 4^\circ\text{C}$ for $\sigma = 0.7$ and to $\sim 1^\circ\text{C}$ for $\sigma = 0.9$. Thus substantial forcing towards warming over this 100–200-Myr period is indicated if σ is less than about 0.7. An estimate¹³ for σ of between about 0 and 0.5 thus supports such a forcing, but the steady-state assumption used for the present crust-mantle exchange must be treated with caution in view of the possibility of transients as demonstrated here. There is evidence for CO_2 degassing in areas of magmatic and metamorphic activity associated with plate boundaries². Metamorphism of carbonates on subducting slabs is probably the principal source of such outgassing (R. Berner, personal communication). More study of the geochemistry of subducting slabs and the outgassing of CO_2 is required, but it appears quite likely that our present carbon cycle is in a transient resulting from an increase in β .

In reality, cooling has occurred during the past 100 Myr (ref. 16). A decrease in the rate of sea-floor generation and an increase in weathering from the increase in continental area have caused a lower degree of degassing, resulting in a forcing towards cooling of $5\text{--}12^\circ\text{C}$ (refs 2, 3, 5). This could have been balanced by a roughly concurrent forcing-towards-warming, apparent here as an increase in β . But if another, independent, forcing-toward-cooling of about the same absolute magnitude arose from the evolution of angiosperm-deciduous ecosystems⁶, the result could have been the net cooling observed. Shifts in continental positions are an additional contributory factor to the observed cooling¹⁷. In any case, it is likely that the persistence in the recent past of substantial deep-ocean carbonate burial, considered as an isolated effect, is a warming factor today. Given the close relationship in the glacial/interglacial cycle between climate and CO_2 (ref. 18), the substantial deposits of carbonates on the subducting slabs may be contributing to atmospheric CO_2 sufficiently to avoid continuous glacial conditions on the Earth.

I thank D. D. Marais, J. Kasting and S. Richardson for participating in early probes into these ideas; K. Caldeira, M. Rampino and R. Berner for discussions; L. Kump for comments on the manuscript; and B. Wilkinson and J. Walker for a preprint. Research support provided by NASA.

Received 24 October 1988; accepted 6 January 1989.

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Implosive earthquakes at the active accretionary plate boundary in northern Iceland

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Non-double-couple earthquake focal mechanisms imply a mode of seismic failure other than simple shear, and movements perpendicular to the fault plane. Reports of such events are rare and it is controversial whether such failure can occur at depth in the Earth. Following reports of such earthquakes from the Hengill and Reykjanes volcanic systems on the accretionary plate boundary in Iceland^{1–3}, a seismological survey was conducted in the Krafla system, Iceland, to explore whether such earthquakes occur elsewhere along the boundary. Here we report the observation of a mixed suite of focal mechanisms at Krafla, including shear events, explosive tensile-crack events such as were observed at Hengill and Reykjanes, and implosive events. These last represent cavity collapse and constitute an entirely new class of earthquake. The Krafla system had undergone a crustal spreading episode^{4–10} during the decade before the observations were made, and our results indicate that immediately after such an episode the accretionary plate boundary is characterized by a heterogeneous stress regime, with shear, extensional and compressional sources in close juxtaposition.

In 1985, the earthquake activity associated with the Krafla volcanic system, on the accretionary plate boundary in Iceland, was investigated with a 30-station, temporary seismometer network (Fig. 1). The impetus for this work came from recent, similar experiments in other parts of the spreading plate boundary in Iceland, where small earthquakes with non-double-couple focal mechanisms were discovered^{1–3}. In the Hengill area (inset, Fig. 1), about half of the 178 events studied were of tensile-crack type, and at Reykjanes a few similar events were observed. One of the goals of the Krafla experiment was to test the hypothesis that such events characterize the spreading plate boundary in general.

Very accurate hypocentral locations were obtained for 100 small earthquakes ($-2.0 < M_t < 2.1$, where M_t is the coda duration magnitude, normalized to the surface wave magnitude M_s) in the depth range 1–4 km. 25 focal mechanisms were well constrained by P-wave first motions, of which 17 did not violate a double-couple, shear interpretation, three were non-double-couple, explosive, and fitted a tensile-crack interpretation similar to the Hengill and Reykjanes events^{1–3}, and five were non-double-couple and predominantly implosive. These latter events represent cavity collapse at depths of up to 3.5 km in the Earth's crust. This study provides further evidence for non-double-couple earthquakes. It also indicates an inhomogeneous stress field—in contrast to those from other volcanic systems on the accretionary plate boundary in Iceland, where relatively homogeneous stress fields were revealed^{1–3}—and shows compressional phenomena occurring within a regional extensional environment.

The Krafla volcanic system is a segment of the neovolcanic

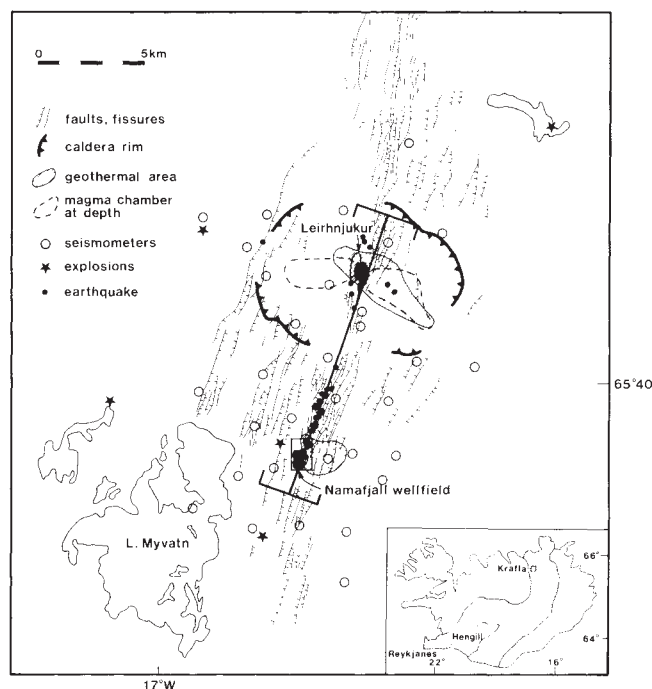


Fig. 1 Tectonic map of the Krafla area, the seismograph network and earthquake epicentres. The outlines of the magma chambers are taken from ref. 4. The bold line corresponds to the section of Fig. 2. Inset shows a map of Iceland. The neovolcanic zone, which marks the locus of the accretionary plate boundary, is shaded, and the locations of the Krafla, Hengill and Reykjanes areas are shown.

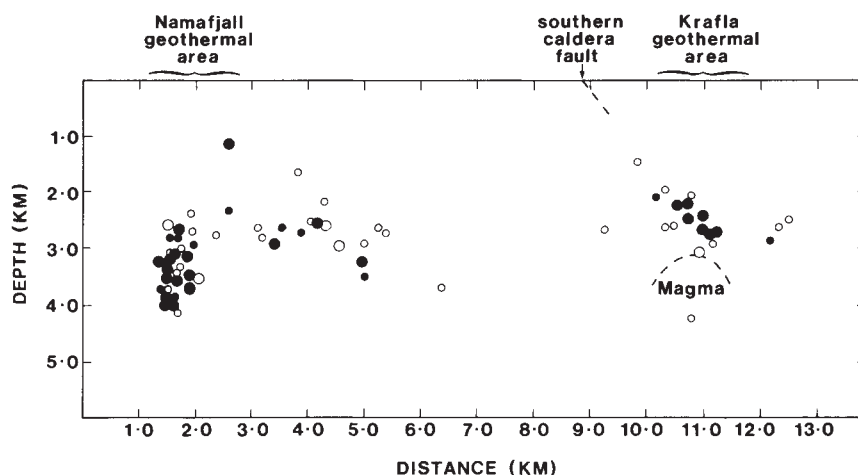
zone in Iceland¹¹ (Fig. 1). It displays a fissure swarm and a central volcano with a caldera 10 × 7 km in size, underlain by a molten body in the depth range 3–7 km (ref. 4). Two separate geothermal areas lie within the system (Fig. 1). In 1975 the central volcano became active and triggered a spreading episode. For several years its magma chamber inflated continually at a rate of $\sim 5 \text{ m}^3 \text{ s}^{-1}$, and deflated rapidly at intervals of a few months^{5–10}. Magma then escaped from the chamber and was intruded along the fissure swarm to form vertical dykes. Up to 8 m of spreading occurred, after which further intrusions could not be accommodated by the altered stress field, and surface magma eruptions occurred¹⁰.

At the time of this experiment, the area was volcanically quiescent, and our aim was to study the continuous seismicity associated with the geothermal areas. The majority of earthquakes were located within these areas (Fig. 1) and activity was continuous on a daily basis. It extended to 4 km depth beneath the Namafjall geothermal area in the south, and 3 km depth beneath the Krafla geothermal area in the north (Fig. 2).

The majority of events beneath the Krafla area directly overlie the intersection of the molten body with the 2-km-wide central part of the fissure swarm where most of the spreading occurred¹⁰. The events form a zone dipping northerly at an angle of 45°, the surface projection of which lies close to the southern caldera fault (Fig. 2), where minor geothermal activity occurs.

Seismic activity within the Namafjall geothermal area clustered in a narrow 'chimney' 2–4 km deep, directly beneath a producing wellfield (Fig. 2). We conclude that these seismic events are induced by heat mining, and the seismically active volume indicates the extent of the cooling zone^{2,3,12,13}. Of 20 well constrained focal mechanisms derived for this volume, 16 were predominantly of strike-slip double-couple type with variable orientations of the P and T axes (Fig. 3), and two were predominantly compressive and of tensile-crack type, similar to those observed at Hengill^{2,3} and Reykjanes¹ (I and II, Fig. 4a). The crack orientation was NNW, oblique to the orientation of the rift zone. In addition, two focal mechanisms displayed

Fig. 2 SSW-NNE cross-section of the region of Fig. 1, showing hypocentral locations.



predominantly dilatational arrivals, and were therefore implosive and indicative of cavity collapse (III and IV, Fig. 4a). These results indicate that the seismic volume is deforming chaotically and that the stress field is inhomogeneous, in contrast to the Hengill and Reykjanes volcanic systems, where all focal mechanisms were consistent with a uniform extensional stress field.

A third group of events occupy a NNE-trending zone at depths of 2–3.5 km, the locus of recent southerly dyke injections⁷. The four well constrained focal mechanisms obtained for this zone are all non-double-couple, one being of tensile-crack type (III, Fig. 4b) and three displaying cavity collapse (I, II and IV, Fig. 4b).

Reports of non-double-couple earthquake radiation patterns are rare, so that these data must be examined critically. The P waves were impulsive and did not have the emergent character sometimes observed in volcanic regions, and the seismometer polarities were tested using both explosions and teleseisms. Thus the polarities of the data points presented in Fig. 4 are reliable. Distortion of the ray paths by crustal heterogeneity was ruled out by the refraction-shot data, which showed that the crust is laterally homogeneous to within 5% of the average one-dimensional crustal model used for locating the hypocentres. In addition, the non-double-couple events occurred in the same volume as double-couple events. These factors rule out all explanations, other than non-double-couple source processes, for our observations.

In this spreading environment it is to be expected that the collapsing cavities are cracks orientated parallel to the fissure swarm. The expected radiation pattern of such an event would be omni-dilatational¹⁴. All events show one or more compressions, however, and in Fig. 4 we show suggested positions for small circular nodal lines that do not violate the data. The seismic moment density tensors of the dilatational events range from $[-1, -1, 0.7]$ to $[-1, -1, 0.3]$. Such sources imply cavity collapse along the edges of the cracks. The event shown in Fig. 4b(II) could be fitted only by small circular nodal lines, with the compressive field forming the continuous belt, and such a source represents crack closure. By analogy with source mechanisms that have been proposed to explain the tensile-crack radiation patterns^{2,3}, the compressive component might be explained by an increase in pore pressure in the crack at the instant of collapse. Such a mechanism would generate small circular nodal lines. An alternative explanation for the compressive field is that it may be generated by a shear component of movement in the source mechanism, in which case the nodal lines would not be small circles.

The non-double-couple events do not exceed magnitudes of $M_t = 0.7$, whereas the double-couple events range in magnitude up to $M_t = 2.1$. The very close correlation of the earthquakes with surface heat loss, and analogies drawn with the Hengill area, are convincing evidence that these events are induced by

thermal cracking^{2,3,12,13}. The total volumetric component of the earthquakes may be calculated, and compared with the volume contraction resulting from heat loss^{2,3}. It is found that the seismic volumetric component is two orders of magnitude smaller than the cooling volumetric component. This result indicates that the majority of the volume contraction resulting from the surface heat loss is accommodated aseismically, a conclusion also reached for the Hengill geothermal area.

The volumetric contraction resulting from surface heat loss reduces the confining stress in the geothermal heat source. In many geothermal areas, this results in continuous, small-magnitude earthquake activity that releases stress in accordance with the regional field^{2,3,15–18}. It is to be expected that at the accretionary plate boundary this stress field is uniform and extensional, and that tensile-crack opening occurs^{2,3}. This is observed at Hengill^{2,3} and Reykjanes¹. The heterogeneous suite observed at Krafla contrasts with this, probably because of the recent spreading episode, which released the extensional stress field. No such rifting or volcanism is reported for the Hengill and Reykjanes areas for 200 and 700 yr respectively^{19,20}.

The observations from the Krafla volcanic system provide further evidence that small-magnitude non-double-couple seismic failure characterizes the accretionary plate boundary in

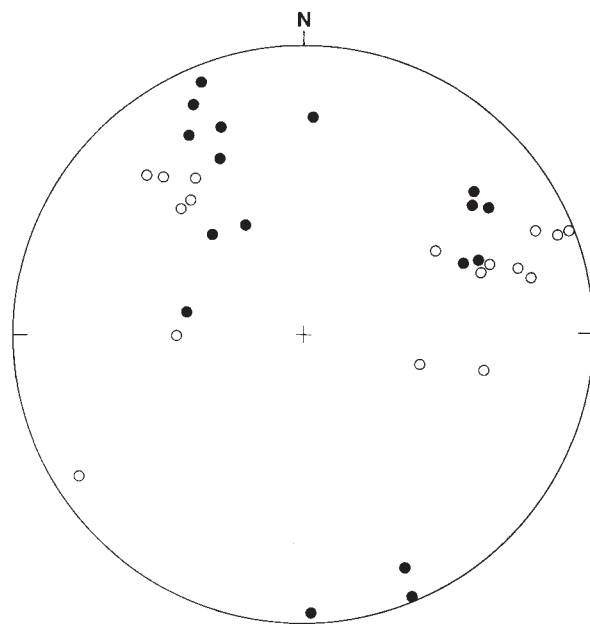


Fig. 3 Stereographic plot of the pressure (○) and tension (●) axes of the double-couple events. These are related to (though not equivalent to) the axes of greatest and least principal stress respectively.

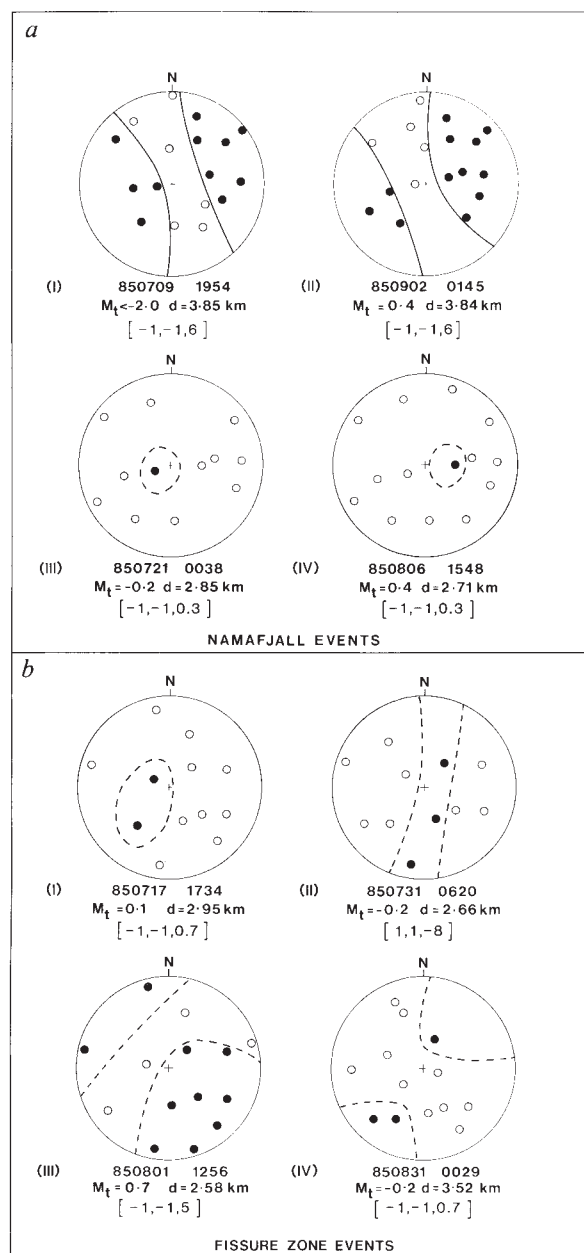


Fig. 4 Non-double-couple focal mechanisms obtained for events beneath the Namafjall geothermal area and along the fissure zone. Filled circles indicate compressions and open circles indicate dilatations. These are upper focal-sphere plots in stereographic projection. Dashed lines are suggested positions of small circular nodal lines. Event captions indicate origin time, local magnitude (M_t), depth (d) and approximate seismic moment density tensor.

Iceland. The Hengill and Reykjanes observations show that an extensional-stress regime may develop during long periods of quiescence between spreading episodes, and that both double-couple and non-double-couple extensional seismic failure occurs. In contrast, the Krafla observations indicate that the immediate post-spreading phase in the accretionary tectonic cycle is characterized by a chaotic stress field, with double-couple events of variable orientation, and both extensional and compressional non-double-couple sources in close juxtaposition.

This project was financed by the Natural Environmental Research Council (who also lent Geostore field-recording equipment), the National Energy Authority, Iceland, and a grant from the Icelandic Science Fund to the Science Institute, University

of Iceland. Larus Bjarnason, Bob Jones, Mike Smith and Dave Stevenson gave invaluable assistance. Roger Bilham improved the manuscript.

Received 27 October 1988; accepted 17 January 1989.

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Latest Proterozoic plankton from the Amadeus Basin in central Australia

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The plankton of the Proterozoic is preserved mainly as microfossils known as acritarchs, most of which are considered to be cysts of eukaryotic algae. In recent studies of the diversity of Proterozoic and early Palaeozoic acritarchs, a gradual increase in diversity from the Middle into the Late Proterozoic has been shown to be followed by a sharp decrease in diversity in the latest Proterozoic (Ediacarian or Vendian) and then a rise again in the Early Cambrian^{1,2}. These observations have been interpreted in terms of the evolutionary diversification of eukaryotic algae and the ecological effects of the pre-Ediacarian glaciation. Occasional discoveries of complex acritarchs in Ediacarian rocks^{3–6} have raised some doubts about these interpretations, but more recent work seems to have supported this view⁷. Here we report the discovery of a diverse assemblage of large and morphologically complex acritarchs from the Ediacarian age upper Pertatataka Formation in the Amadeus Basin of central Australia. This, with a recent report of similar fossils from the upper Sinian of China⁸, provides a significant new perspective on the history of the plankton. It is now necessary to suggest an earlier radiation, at least in off-shore environments, or to question the reality of the postulated decrease in diversity. In addition, there may well have been an extinction event, late in the Ediacarian. Neither of these phenomena has been recognized previously.

The Amadeus Basin is intracratonic (Fig. 1) and has a sediment fill ranging in age from about 800–900 Myr BP to late Palaeozoic. Dykes in the basement are unconformably overlain by the basal sandstone of the basin and are dated (Rb/Sr on separated minerals) at 897 ± 9 Myr BP providing a maximum age for the sedimentary sequence⁹. The Pertatataka Formation overlies the upper of two Proterozoic glacial sequences and underlies the Arumbera Sandstone which in its lower part contains fossils of the soft-bodied Ediacara fauna (Fig. 2). The upper Arumbera Sandstone contains abundant and diverse trace

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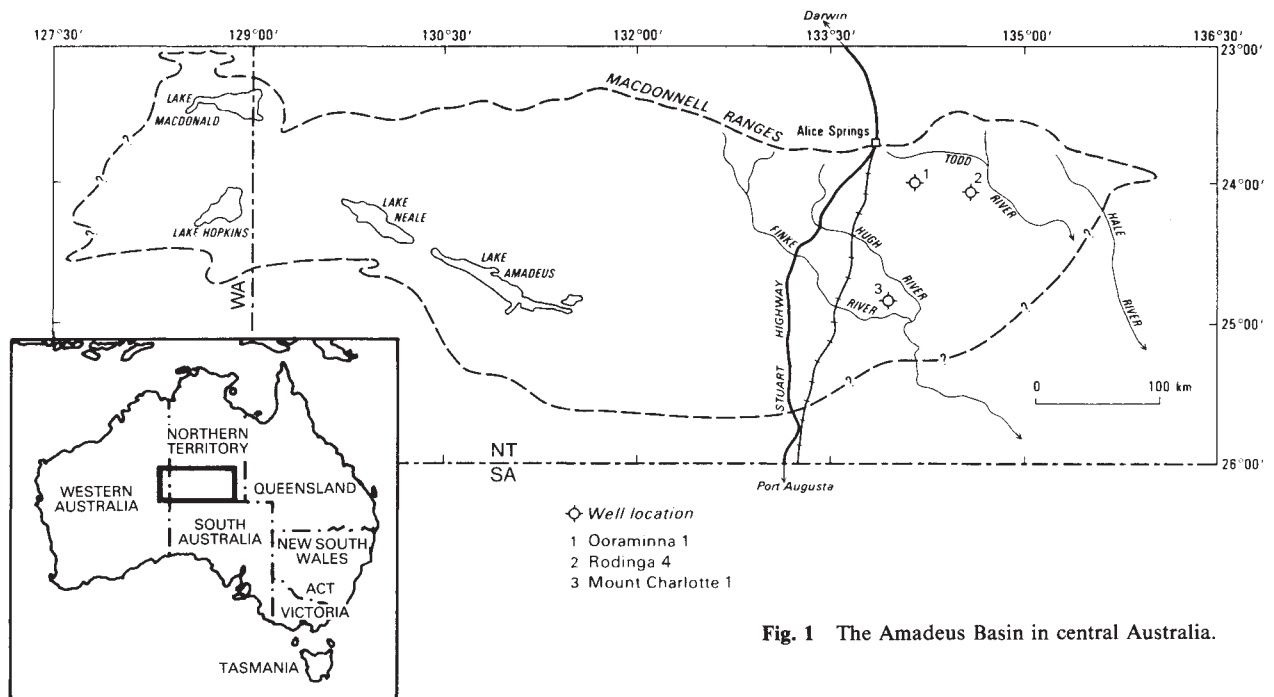


Fig. 1 The Amadeus Basin in central Australia.

fossils like those in Early Cambrian rocks world-wide^{10,11}, and it in turn is overlain by a carbonate with small shelly fossils of Atdabanian (mid Early Cambrian) or slightly greater age¹². We can be confident that the Pertatataka Formation is Late Proterozoic, and lithostratigraphic correlations with the nearby Adelaide Geosyncline establish an Ediacarian age^{13,14}.

The Pertatataka Formation is poorly exposed and for this and other reasons has been little studied, but recent work shows it to be a sequence of distal turbidites. It is made up primarily of red and green shales and siltstones, with minor, thin sandstone beds. The lack of conglomerate and coarse-grained channel sands, the preponderance of base cut-out sequences and the fact that the coarsest grain size is fine sand, are considered to indicate that the turbidites were deposited in an outer submarine fan to basin plain environment¹⁵. The material used in this study came from oil exploration and stratigraphic drill-holes Ooraminna number 1, Rodinga number 4 and Mt Charlotte number 1 (Fig. 1). We have studied about 40 samples from a stratigraphic interval of 140 m in Rodinga number 4, six samples from Ooraminna number 1 and five samples from Mt Charlotte number 1. The diverse assemblage is restricted to the upper part of the Pertatataka Formation, which occurs in only the first two of these drill holes.

The lower Pertatataka Formation contains what could until now have been regarded as a typical Ediacarian assemblage of acritarchs, consisting of 13 taxa of simple spheroidal forms (the number of taxa must be considered tentative until the taxonomic systematics of this assemblage has been published). The assemblage is not known to include any spinose acritarchs. The spheroidal forms include common species of *Trachysphaeridium*, *Kildinosphaera*, *Protoleiosphaeridium*, *Stictosphaeridium* and *Leiosphaeridia*, as well as some new species. Examples of *Kildinosphaera* are particularly abundant. One sample has very abundant examples of acritarchs comparable to *Bavlinella*, with growth sequences like those described for *Sphaerocongregus*¹⁶.

Possibly as many as 40 species are recognizable in the upper Pertatataka assemblage (again, the number must be treated with caution at this stage). Many of the simple spheroidal forms found in the lower assemblage are also present here, and there are two distinctive new spheroidal forms. One has 12–20- μ m cells inside an 80–200- μ m-wide vesicle. A second has numerous cells within 150- μ m-wide vesicles. Some well preserved filamen-

tous microfossils have been found in samples from Rodinga number 4. Most are fragmentary and were probably transported from a nearshore environment. They are referable to well known taxa such as *Eomycetopsis*, *Siphonophycus*, *Oscillatoropsis* and the helically coiled *Obruchevella*.

Of most interest are the large and lavishly ornamented forms (Fig. 3). These are abundant and contain more than 50% of all taxa recognizable in the younger assemblage. Most abundant is a form we identify as a new species of *Cymatiosphaeroides*, an acritarch with numerous thin spines between the main vesicle and an outer membrane. This form is about 180 μ m wide. There are also large acritarchs (about 180 μ m wide) with abundant hair-like spines up to 40 μ m long, which we regard as a new species of *Comasphaeridium*. Next most abundant in the assem-

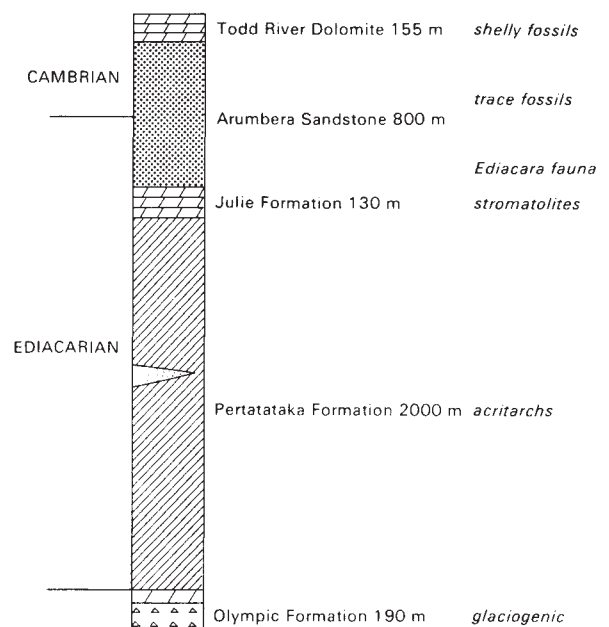


Fig. 2 Generalized stratigraphic section for the NE Amadeus Basin.

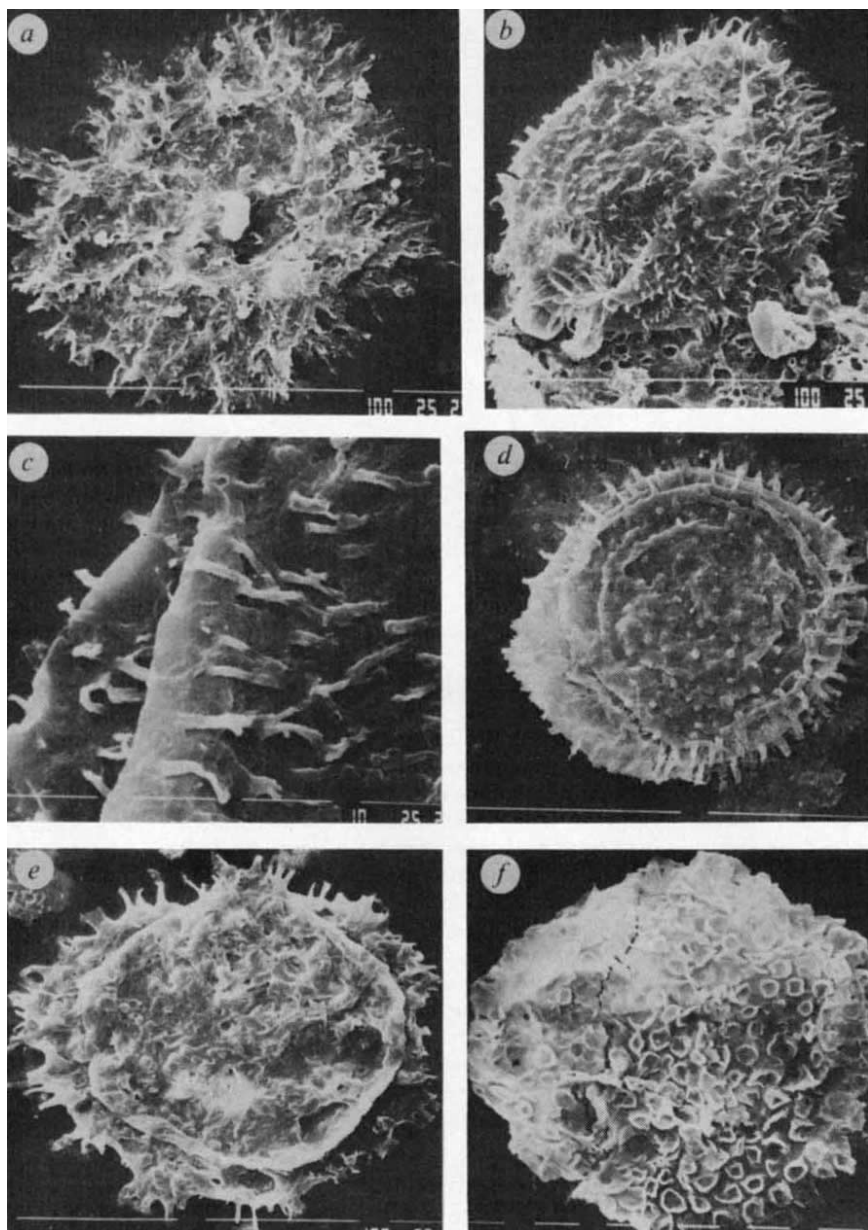


Fig. 3 Acritarchs from the Ediacarian Pertatataka Formation from the indicated depths in BMR drill hole Rodinga number 4 in the Amadeus Basin, central Australia. *a*, Large vesicle with frequently branched processes (34.50–34.76 m). *b*, *Trachyhystriosphera* (34–35 m). *c*, *Skiagia* (48.38–48.67 m). *d*, *Gorgonisphaeridium* (53.86–54.14 m). *e*, Unnamed form (52.16–52.50 m). *f*, Unnamed form bearing tube-like processes opening at the outer end but not communicating with interior of the vesicle (48.92–49.24 m). Scale bars, 100 µm for *a*, *b*, *d* and *e*, and 10 µm for *c* and *f*.

blage are very large vesicles with hollow spines; we tentatively identify these as new species of *Baltisphaeridium* and *Trachyhystriosphera*. There appear to be several different species present; one form has vesicles up to 240 µm in width and abundant broad-based spines up to 15 µm long. Another has vesicles about 100 µm wide with numerous slender, curved spines up to 40 µm long. Also present is a previously undescribed type of acritarch, with vesicles about 200 µm wide and with two kinds of spines, each confined to discrete areas of the vesicles: one kind is thick and isolated like those of *Baltisphaeridium*, the other thin and hair-like as in *Comasphaeridium*.

Other spinose acritarchs are much less common. Perhaps the most distinctive is a previously undescribed very large acritarch with a vesicle 400 µm wide and numerous thick frequently branched processes up to 60 µm long. Amongst the other spinose forms are some in which the spines have thickened tips, another with broad-based spines like those of *Comasphaeridium*, and a third with blunt-tipped spines. There is also a form with short, branched spines possibly referable to *Gorgonisphaeridium*. Acritarchs up to more than 300 µm wide with short, funnel-

shaped processes that open into the vesicles are present in low abundance. We treat these as a new, very large species of the genus *Skiagia*. A rare and completely new form has vesicles 100 µm wide with short, broad, pipe-like processes (Fig. 3*f*).

Several of these genera have previously only been recognized in Cambrian and younger deposits^{17–20}, although the forms we have discovered are very much larger than their Palaeozoic equivalents. This assemblage is distinctive and different at least at the specific level from younger examples. It is a matter of taxonomic judgement as to whether to include these newly discovered very large acritarchs in genera previously defined for much smaller forms; we have chosen to do so because in all respects other than size our specimens fit the generic diagnoses. Some of the Pertatataka acritarchs also resemble large spinose forms such as *Cymatiosphaeroides* and *Trachyhystriosphera* that are rare components of older Late Proterozoic assemblages²¹.

Many examples of Ediacarian acritarch assemblages have been described and almost without exception they consist of unornamented spheroidal forms (leiosphaerids) often accom-

panied by macroscopic algae (vendotaenids). A recently described example is that of the Arcoona Quartzite Member, a correlative on the Stuart Shelf of South Australia of the middle Pertatataka Formation of central Australia⁷. However, the new discoveries in the Doushantou Formation of the Yangtse Platform^{6,8} and the upper Pertatataka Formation indicate that there was an assemblage of diverse and complex plankters at this time. We consider that there are at least two possible reasons why this assemblage has not been recognized previously: (1) it was not as readily preserved as older and younger assemblages; (2) it lived in an off-shore marine environment that is poorly represented in the Ediacarian geological record. Evidence for the first explanation is the preservation of the assemblage in cherts of the Doushantou Formation⁶ but not in interbedded shales. Cherts are well known to be an exceptionally effective medium for the preservation of microfossils, but acritarchs are usually found in shales and siltstones as well as in cherts. Second, few turbidites of definite Ediacarian age seem to have been searched for acritarchs. 'Proterozoic sequences which have been micropalaeontologically investigated so far usually represent shallow marine depositional conditions'¹. The environment of deposition of the Doushantou Formation is poorly known; pseudomorphs of gypsum are reported from dolomite in the lower part of the formation and ooids from the upper part, suggesting shallow water to emergent environments. The acritarchs come from cherts in flat-laminated silty dolomites in the middle part of the formation, which has been interpreted as having been deposited in a quiet, relatively deep marine environment²². The occurrences in the Pertatataka Formation might be the result of unique preservational circumstances, but they certainly suggest that more attention to turbidites is justified.

Previous authors have postulated a sharp decrease in the diversity of the plankton at the time of the widespread pre-Ediacarian glaciation, with no subsequent diversification until Early Cambrian times. It is now necessary to suggest an earlier radiation, at least in off-shore environments, or to question the reality of the postulated decrease in diversity. In addition, there may well have been an extinction event, late in the Ediacarian: the Ediacarian assemblage is very distinctive and different from all younger assemblages, even though we have chosen to place some of the new species in Palaeozoic genera. Many individual Ediacarian acritarchs are some two orders of magnitude greater in volume than comparable forms of Cambrian age. The Proterozoic history of the plankton is still very poorly known and caution is obviously necessary, but our observations do seem to indicate a previously unrecognized extinction event.

We thank G. Vidal, M. Moczydlowska, A. H. Knoll, J. M. Hayes, E. M. Truswell and R. E. Summons for useful comments.

Received 17 April; accepted 14 December 1988.

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Evolution of seed dormancy

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Palaeozoic fossil conifer cones bearing mature seeds with well developed cotyledonary embryos have been recovered from Permian–Carboniferous strata of North America. These are the most ancient embryos ever discovered within gymnosperm seeds^{1,2}. In contrast to the pteridophytic ancestors of seed plants and other major groups of Palaeozoic gymnosperms^{3,4}, these embryos indicate that there was a significant delay between fertilization and seed germination in the earliest conifers. We suspect that this post-zygotic quiescence may be a first step in the evolution of seed dormancy.

Although gymnosperm ovules from Palaeozoic sediments sometimes contain well preserved gametophyte tissue^{5,6}, embryos are exceedingly rare^{1,2}. Among the hypotheses attempting to account for the paucity of embryos are two that propose that the post-zygotic development in the earliest gymnosperms occurred as a rapid and continuous sequence^{3,4}. Modern, and presumably ancient, pteridophytic reproduction is characterized by continuous development from spore germination through to fertilization, embryogeny and sporeling growth^{7,8}, but the embryos of most modern seed plants have a quiescent period before seed germination and seedling establishment. Moreover, many seeds remain dormant until a physical or physiological barrier has been overcome^{9,10}. Dormancy is more common in flowering plants than in gymnosperms, but some degree of quiescence characterizes living seed plants in virtually all the main climatic zones^{11–14}. Both mechanisms coordinate seed germination and seedling establishment with favourable environmental conditions.

This report of well developed embryos in Palaeozoic conifer seeds may provide evidence for the evolution of quiescence. The seeds are preserved within the cone of a walcchian conifer (Fig. 1a) from limestone deposits of either the uppermost Pennsylvanian or lowermost Permian, near Hamilton, Kansas^{15,16}. Mature ovules and seeds are 5.5–7 mm long with a well developed integument (Figs 1a and 2) and an open micropyle (Fig. 2). The nucellus is thin and contains a large megaspore membrane. Distally the nucellus forms a prominent pollen chamber with a differentiated apical beak (Fig. 2).

Among the 34 seeds present in the Hamilton cone there are cellular megagametophytes in six, and well developed embryos (Figs 1b and 2) in four. These numbers are consistent with the low percentage of mature seeds which typically develop in normal cones of modern conifers^{17,18}. The fossil seeds show no evidence of larval or fungal damage, and their range of structural variation is consistent with normal developmental variation among healthy seeds of gymnosperms^{19,20}. In the Hamilton seeds, cellular gametophyte tissue is preserved in the centre of the megaspore interior. It is unzoned and forms an elongated ellipse with an irregular periphery.

The most mature embryo (Figs 1b and 2) is about 2.5 mm long and slender. There are about six cotyledons surrounding the epicotyl, and they are tightly appressed to one another (Figs 1b and 2). The radicle lies adjacent to the pollen chamber, with the cotyledons extending towards the chalaza (Fig. 2). Tracheid maturation is incomplete, but there is a slender zone differentiated at the centre of the embryo (Fig. 1b) that may represent procambium. As in modern conifer seeds, the embryo is separated from the gametophyte tissue by a narrow, well defined corrosion cavity (Fig. 1b). Large cells at the tip of the radicle seem to be similar to the suspensor tissue of extant conifer seeds¹⁹.

These are the most ancient seeds known to contain cotyledonary embryos, and they are evidence that the embryogeny

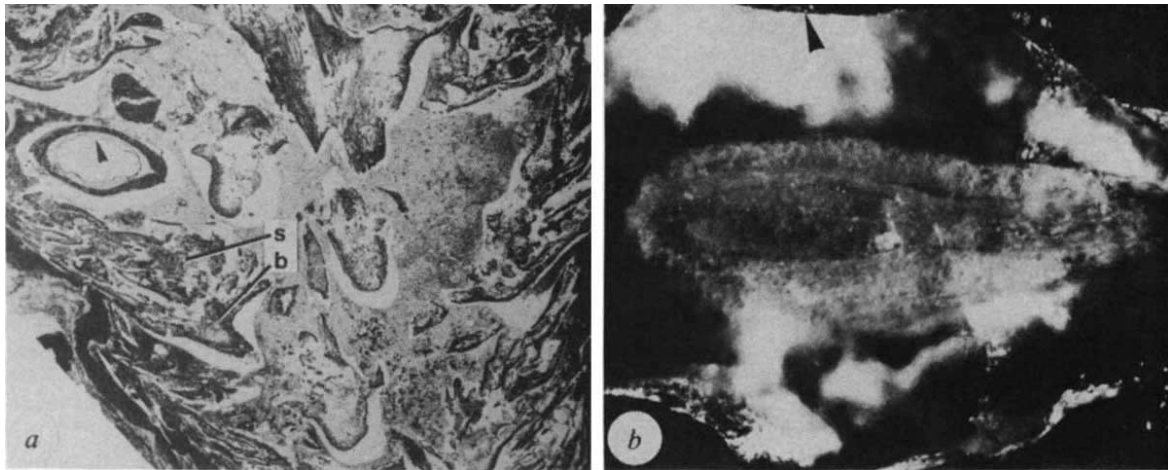


Fig. 1 Walchian conifer cone, bearing seeds with cotyledonary embryos. *a*, Nonmedian longitudinal section of cone showing bracts, fertile shoots and seed (*B*, bract; *S*, shoot) (cone M1613, BL number 32×5); *b*, Reflected light view into the seed cavity of seed in *a*. Embryo with cotyledons at left and radicle at right within cellular megagametophyte tissue (×55). Arrows in *a* and *b* indicate comparable positions on megaspore membrane.

and the bipolar growth of modern gymnosperms characterized primitive Palaeozoic conifers. They also indicate that quiescence before seed germination may have initiated the evolution of seed dormancy. Perhaps more significantly, these seeds provide insight into the selection pressures and developmental mechanisms that could have been associated with the evolution of seed dormancy. Like those of pteridophytes, embryos of the most primitive gymnosperms (including conifer ancestors) presumably had continuous development from the zygote to seedling establishment^{3,4}. In the relatively moist lowlands inhabited by the most ancient gymnosperms²¹ environmental conditions were probably conducive to seedling establishment most of the time, and immediate germination of seeds probably did not pose a significant reduction in fitness. Immediate germination also would dramatically reduce the probability of seeds with well developed embryos becoming fossilized. But in environments which are more arid or only periodically moist, unregulated germination would lead to the death of a large percentage of seedlings. Under such conditions, delay of seed germination until the onset of the appropriate environmental conditions could impart a significant selective advantage.

As we have suggested here, conifers may have been the most

ancient seed plants to display quiescence before seed germination, a possible adaptation for coordinating seed germination with intermittent wetness. We envisage that quiescence, together with specialized features of the vegetative organs, may have dramatically increased physiological tolerance to water stress. The first occurrence of quiescence in these ancient conifers is concordant with the initial establishment of extensive plant communities on relatively dry extra-basinal slopes^{23,24} and provides evidence that the origin of seed dormancy contributed to a phenotypic breakthrough^{24,25} that facilitated colonization of previously unforested habitats. This emphasizes the role of evolutionary ecology in significantly increasing the numbers and diversity of Palaeozoic conifers as major drying trends produced widespread aridity in the Permian.

This work was supported by the National Science Foundation, the Kansas Geological Survey and Ohio University.

Received 3 October; accepted 21 December 1988.

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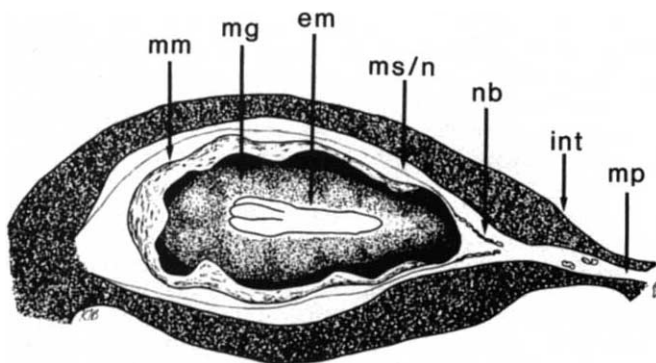


Fig. 2 Reconstruction of seed with embryo in Fig. 1. Based on cone M1613, BL number 17–32. Note pollen in micropyle (*em*, embryo; *int*, integument; *mg*, megagametophyte; *mm*, megaspore membrane; *mp*, micropyle; *ms/n*, megasporangium/nucellus; *nb*, nucellar beak).

Benign familial neonatal convulsions linked to genetic markers on chromosome 20

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Recurrent seizures, commonly known as epilepsies, occur in 1.7% of the general population by age 40¹. The factors that initiate or underlie seizures are not well understood, but trauma, infectious disease and genetics have been implicated. An understanding of the molecular basis of seizures would shed light on the basic mechanisms of neuronal homeostasis and allow new therapeutic strategies to be explored. Here, we report the mapping of an epilepsy gene to a specific chromosomal region, on the basis of cosegregation of two closely-linked DNA markers with a form of epilepsy known as benign familial neonatal convulsions (BFNC², #12120 in ref. 3). The linked markers confirm the genetic basis and autosomal dominant inheritance of this trait, and localize the gene causing BFNC in this family to the long arm of chromosome 20. This regional placement is the first step towards the isolation of a gene involved in neuronal activity in the human brain.

The segregation pattern of BFNC has indicated an autosomal dominant mode of inheritance with high penetrance. Including the original description of this type of seizure by Rett and Teubel in 1964⁴, 24 separate pedigrees have been identified⁵⁻¹³. Diagnosis of BFNC is based on inheritance, the exclusion of other causes for neonatal seizures, and the developmental pattern of the seizures, which typically start within the first week of life and end spontaneously by six months of age. Subsequent neurological and intellectual development is normal, although a

minority (about 10%) of individuals have seizures later in life⁵.

We undertook a genetic linkage study of a large, four-generation family (Fig. 1) that included 19 individuals who had documented neonatal or infantile seizures and had met the specific diagnostic criteria for BFNC¹⁴. Because accurate phenotypic characterization of family members is critical for the success of this approach, the results of laboratory tests for other possible causes of neonatal convulsions were examined for recent generations; all were negative¹⁴. Pyridoxine dependency was ruled out as the cause of seizures in this family because of the dominant mode of inheritance, the ability of standard anticonvulsants to control the seizures and the absence of mental retardation.

Linkage analysis was performed with the program LINKAGE¹⁵. We assumed autosomal dominant inheritance of a BFNC gene with a penetrance of 0.9. This conservative assumption of high but incomplete penetrance is supported by published accounts of four families in which an unaffected person has an affected child and also an affected sibling or parent, and by the estimated segregation ratio of 42% in 208 siblings and offspring of probands in 17 BFNC families^{8,10,16,17}. Two polymorphic DNA marker loci, CMM6 (D20S19, ref. 18) and RMR6 (D20S20, ref. 19), were found to be tightly linked to the disease locus, giving maximum lod scores (log likelihood ratios) of 3.12 ($\theta = 0.003$) for RMR6 and 2.87 ($\theta = 0.039$) for CMM6; the score for RMR6 corresponds to a likelihood ratio of >1,000/1 in favour of linkage (Table 1).

Joint analysis of the inheritance pattern of the three loci greatly increased our confidence that the two markers are linked to the BFNC gene. In our panel of 59 reference families²⁰, CMM6 and RMR6 had a maximum lod score at a recombination frequency of 1.6%; when a recombination fraction of 0.016 was assumed for the interval between the two markers in joint analysis with the BFNC locus, the likelihood ratio supporting linkage of the BFNC gene to the two marker loci increased to >500,000/1 (lod score of 5.64, Table 1). The total number of markers tested was 96, and therefore significance for linkage would require a maximum lod score of at least 5.0 (ref. 21). As there are no obligate recombinants in this family we were unable to order the disease locus with respect to the two markers. Because recombinants might be found in a larger sample,

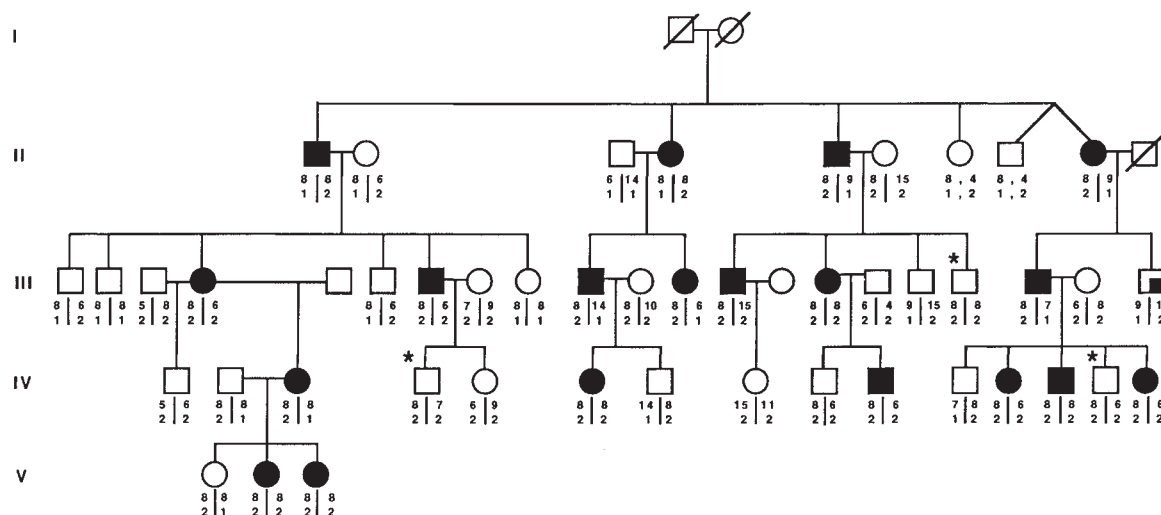


Table 1 Lod scores (multipoint and pairwise) between BFNC and three DNA markers in a five-generation pedigree

Marker locus	Lod score at recombination fraction of					
	0.00	0.05	0.10	0.20	0.30	0.40
CMM6-RMR6	5.64	5.50	5.20	4.20	2.90	1.40
RMR6	3.12	3.05	2.88	2.32	1.57	0.69
CMM6	2.87	2.92	2.83	2.36	1.63	0.76
MS1-27	-9.11	-3.51	-2.33	-1.12	-0.49	-0.15

Lod scores have been calculated with penetrance = 0.9. When penetrance was varied from 0.70–0.95, maximum lod scores ranged from 5.98–5.02.

however, a confidence interval with boundaries defined by a tenfold decrease in likelihood (the 1-lod confidence interval²²) must be considered. This boundary for the BFNC gene is found at a recombination frequency of 15% on either side of the two tightly linked markers.

The two DNA marker loci were mapped to chromosome 20 by linkage in reference families with a third DNA marker locus, MS1-27 (D20S4, ref. 23). The MS1-27 locus has been physically mapped to human chromosome 20 using somatic cell hybrids²⁴, and *in situ* hybridization has confirmed that chromosomal assignment and provided independent, subregional localization of the MS1-27 locus to 20q13.2 (ref. 25). The CMM6 locus gave a lod score of 4.41 with the MS1-27 locus at a recombination frequency of 32.6%, establishing linkage.

We conclude that a mutation at a single genetic locus, located on the long arm of chromosome 20, strongly predisposes individuals in this family to benign neonatal convulsions. Although no obligate recombinants are evident in Fig. 1, the three unaffected individuals indicated by asterisks would be inferred in the linkage analysis to carry the mutation. Without additional information, we cannot determine whether these three subjects reflect recombination events that have occurred between the seizure locus and the marker loci, or reduced penetrance of the BFNC allele.

The next step will be to identify candidate genes from the region of chromosome 20 that seems to harbour the BFNC mutation. Genes that could be considered probable candidates on the basis of their expected physiological role can now be critically tested by determining whether they map to the same region as BFNC; those that map elsewhere can be excluded. For example, a potential candidate for the BFNC mutation might have been the gene encoding glutamate decarboxylase, an enzyme that converts glutamate to the neurotransmitter inhibitor gamma aminobutyric acid; but the glutamate decarboxylase gene is known to reside on chromosome 2²⁶.

Our observation of genetic linkage not only defines a location, but also confirms the single-gene origin of BFNC in one family. There are few clinical grounds for suspecting that different mutant genes are responsible for BFNC in other families, but they do include an impression that the development of non-febrile seizures after six months of age may be restricted to certain pedigrees. The issue of genetic heterogeneity can be resolved by testing additional families with the two markers described here. Attention then can be directed towards a search for the gene and an explanation for the distinctive developmental pattern of seizures in the BFNC syndrome.

The authors wish to acknowledge the technical assistance of M. Hoff, C. Martin, R. Myers, B. Ogden and T. Sears, and to thank I. Bjerre and J. Zonana for their interest in initiating the collection of family material for later tests of heterogeneity. I. Leppik, W. A. Hauser and S. S. Rich provided helpful discussions. R. Foltz edited the manuscript. This work was supported in part by a grant from the NIH-NINCDS to the Comprehensive Epilepsy Program, University of Minnesota. R. W. and J.-M.L. are Investigators at the Howard Hughes Medical Institute.

Received 24 October 1988; accepted 9 January 1989.

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Arginine vasopressin enhances pH_i regulation in the presence of HCO_3^- by stimulating three acid-base transport systems

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Growth factors raise intracellular pH (pH_i) by stimulating Na^+/H^+ exchange in the absence of HCO_3^- (refs 1–4). In mutant cells that lack the Na^+/H^+ exchange activity, this alkalization does not occur, and the cells do not proliferate without artificial elevation of pH_i ^{5,6}. It has therefore been widely suggested that an early pH_i increase is a necessary signal for mitogenesis. In the presence of HCO_3^- however, growth factors fail to raise pH_i in A431 cells⁷, renal mesangial cells⁸ and 3T3 fibroblasts⁹. In mesangial cells, arginine vasopressin (AVP) raises pH_i in the absence of HCO_3^- , but lowers it when HCO_3^- is present; growth is stimulated under both conditions⁸. We report here that, in the presence of HCO_3^- , AVP stimulates two potent HCO_3^- transporters, as well as the Na^+/H^+ exchanger. These are the Na^+ -dependent and Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchangers. Our results indicate that AVP causes acidification in the presence of HCO_3^- because, at the resting pH_i , it stimulates Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange (which lowers pH_i) more than it stimulates the sum of Na^+/H^+ exchange and Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchange (both of which raise pH_i). The stimulation of three acid-base transporters by the growth factor AVP greatly enhances the ability of the cell to regulate pH_i .

We examined the effect of AVP in the presence of HCO_3^- on the two acid-extruding transporters previously identified in mesangial cells^{10,11}: the Na^+/H^+ exchanger and the Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchanger. Figure 1a illustrates the effect of AVP on the ability of cells to recover from an acute intracellular acid load imposed by an $\text{NH}_4^+/\text{NH}_3$ prepulse¹². The application

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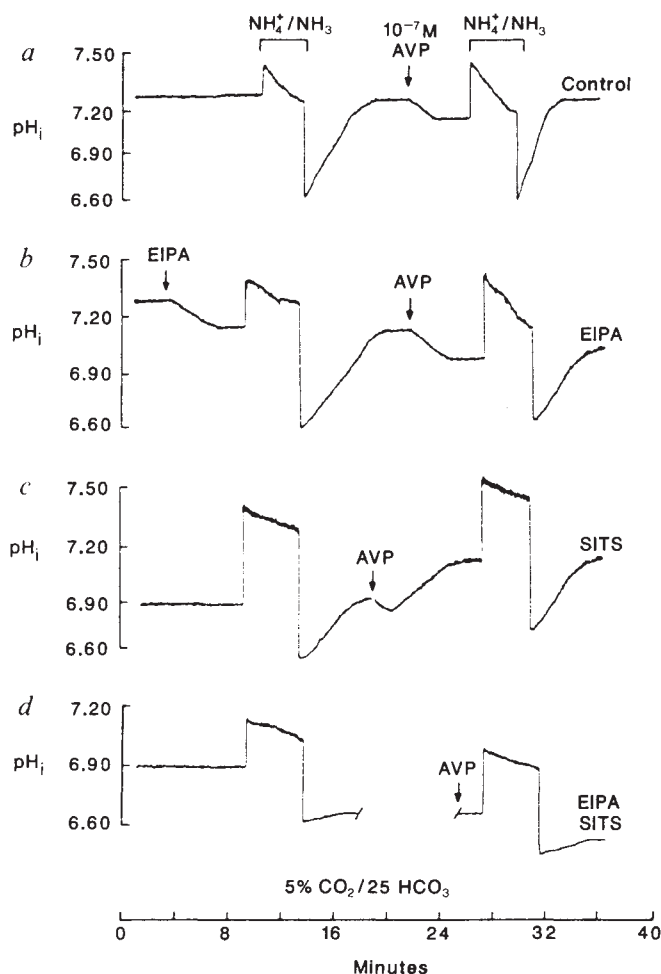


Fig. 1 Effect of 10^{-7} M AVP on the recovery of pH_i from an NH_4^+ -induced acid load. For each of the four protocols, the cells were acid-loaded twice by applying and then withdrawing a solution containing 20 mM NH_4^+ (standard HCO_3^- solution (see below) with 20 mM NH_4^+ replacing 20 mM Na^+), first in the absence and then in the presence of AVP. We verified that, in the absence of AVP, the pH_i recovery rates from two consecutive NH_4^+ pulses were within $7 \pm 6\%$ (NS) of each other (data not shown). *a*, Control in absence of EIPA and SITS ($n = 14$). *b*, With EIPA ($n = 14$). EIPA (50 μ M) was added at the time indicated by the arrow, and was present thereafter. *c*, With SITS ($n = 20$). Because the fluorescence of SITS interferes with that of BCECF (see Methods), SITS was applied to the mesangial cells for 1 hour before the experiment and then washed off vigorously. Addition of AVP to the SITS-pretreated cells produces the pH_i response normally seen in the absence of HCO_3^- , a transient acidification followed by a sustained alkalinization⁸. Thus, the sustained acidification normally produced by AVP in HCO_3^- (a) requires a SITS-sensitive transporter. *d*, EIPA plus SITS ($n = 14$). Cells were preincubated for 1 hour in SITS and then washed as in (*c*). EIPA (50 μ M) was applied during dye loading and was present thereafter. After EIPA/SITS treatment, the acid extrusion is so slow that pH_i fails to recover beyond ~ 6.7 , and AVP failed to stimulate this slow pH_i recovery.

Methods. Mesangial cells were isolated from glomeruli of young male rats, characterized and maintained in culture as previously described¹⁵. MCs (passage 2–5) were grown on glass cover slips in mesangial cell growth medium consisting of DMEM plus 20% FCS, 5 μ g ml^{-1} insulin, 10 mM L-glutamine, 400 ng ml^{-1} penicillin, 500 ng ml^{-1} streptomycin and 25 mM HCO_3^- . Mesangial cells were rendered quiescent by reducing FCS to 0.5% for 48 h before the pH_i experiments. They were then dye-loaded^{8,16} with 10 μ M of the acetoxymethyl ester of BCECF (2,7-bis(carboxyethyl)-5-(6-carboxyfluorescein) from a 10 mM stock in DMSO for 20 min at 37 °C. The cover slip with cells was placed in a thermostatically controlled cuvette (37 °C) in a Perkin-Elmer LS-5B spectrofluorometer. We alternately excited the dye at 440 and 500 nm, and measured the fluorescence emission at 530 nm. The BCECF fluorescence excitation ratio was calibrated using the high- K^+ /nigericin technique^{8,17}. All experiments were performed in CO_2/HCO_3^- solutions. The standard HCO_3^- solution contained (in mM): Na^+ , 145; K^+ , 5.0; Ca^{2+} , 1.8; Mg^{2+} , 1.0; Cl^- , 122; HCO_3^- , 25; SO_4^{2-} , 1.0; phosphate, 1.0; and glucose, 10, and was equilibrated with 5% $CO_2/95\%$ O_2 at pH 7.40. EIPA was a gift of Dr Burckhard (Frankfurt, FRG).

and subsequent removal of 20 mM NH_4^+ causes pH_i to decrease rapidly. The intracellular pH recovers from this acid load because of the activity of the ethyl isopropylamiloride (EIPA)-sensitive Na^+/H^+ exchanger, which accounts for about one-third of acid extrusion¹⁰, and the SITS-sensitive Na^+ -dependent Cl^-/HCO_3^- exchanger, which accounts for about two-thirds¹¹ (see Table 1 legend). After application of 10^{-7} M AVP, pH_i falls slowly as previously described⁸, and recovery from an NH_4^+ -induced acid load is substantially faster. In 14 experiments, AVP increased the mean rate of pH_i recovery ($d(pH_i)/dt$), at $pH_i = 6.60$, from 77.6 to 145.2×10^{-4} pH units $second^{-1}$ ($P < 0.001$). The total buffering power (β_{total}) was 22.5 and 23.7 mM per pH unit in the presence and absence of AVP respectively. Thus, the net acid-extrusion rate ($J_{ext} = d(pH_i)/dt \times \beta_{total}$) increased from 183 to 326 μ M s^{-1} (see Table 1).

To determine the proportion of the increased J_{ext} that is due to stimulation of Na^+/H^+ exchange, we repeated the experiment in Fig. 1a, but blocked Na^+/H^+ exchange with 50 μ M EIPA (Fig. 1b). Application of EIPA causes a sustained pH_i decrease, indicating that Na^+/H^+ exchange is active in the presence of HCO_3^- . EIPA also slows the pH_i recovery from NH_4^+ -induced acid loads, both in the absence and presence of AVP. Table 1 includes a comparison of results of experiments such as those in Fig. 1a and b. In the absence of AVP, EIPA reduces J_{ext} from 183 to 121 μ M s^{-1} , so that the EIPA-sensitive component of J_{ext} (that is, Na^+/H^+ exchange) is 62 μ M s^{-1} . In the presence of AVP, EIPA reduces J_{ext} from 326 to 205 μ M s^{-1} , so that the EIPA-sensitive component of J_{ext} is 121 μ M s^{-1} . Thus, AVP increases Na^+/H^+ exchange activity from 62 to 121 μ M s^{-1} . Nevertheless, this increment (59 μ M s^{-1}) accounts for less than half the total increase in J_{ext} (142 μ M s^{-1}) elicited by AVP.

We performed the experiment shown in Fig. 1c to determine whether stimulation of the Na^+ -dependent Cl^-/HCO_3^- exchanger accounts for the rest of the AVP-elicited increase in J_{ext} . Mesangial cells were pre-incubated for 1 hour with 0.5 mM SITS, a treatment that permanently blocks both the Na^+ -dependent and Na^+ -independent Cl^-/HCO_3^- exchangers^{10,11}. The mean initial pH_i (6.94 ± 0.04) after SITS pretreatment was significantly lower ($P < 0.001$; $n = 20$) than for matched controls (7.30 ± 0.03), indicating that the acid-extruding Na^+ -dependent Cl^-/HCO_3^- exchanger is more active than the acid-loading Na^+ -independent exchanger in the normal steady state. As shown in Fig. 1c, the pH_i recovery rate from an acid load is substantially slowed by SITS pretreatment, both in the absence and presence of AVP. The data are summarized in Table 1. In the absence of AVP, SITS reduces J_{ext} from 183 to 62 μ M s^{-1} , so that the SITS-sensitive component of J_{ext} (HCO_3^- transport) is 121 μ M s^{-1} . In the presence of AVP, SITS reduces J_{ext} from 326 to 90 μ M s^{-1} , so that the SITS-sensitive component of J_{ext} is 236 μ M s^{-1} . Thus, AVP nearly doubles HCO_3^- -dependent acid extrusion, from 121 to 236 μ M s^{-1} . This underestimates the stimulation of Na^+ -dependent Cl^-/HCO_3^- exchange, because SITS pretreatment also blocks the Na^+ -independent Cl^-/HCO_3^- exchanger.

If the AVP-induced increase in J_{ext} is due to the dual stimulation of Na^+/H^+ exchange and Na^+ -dependent Cl^-/HCO_3^- exchange, then the application of both EIPA and SITS would block the effect of AVP. Cells preincubated with SITS and then exposed to EIPA exhibit little recovery from an acid load, with or without AVP (Fig. 1d). In six other experiments (data not shown) we blocked Na^+ -dependent Cl^-/HCO_3^- exchange by pretreating cells for 1 hour in a medium lacking chloride, and blocked Na^+/H^+ exchange with EIPA. The results were identical to those shown in Fig. 1d. Because the SITS-sensitive component of AVP-stimulated acid extrusion is also Cl^- dependent, it is not mediated by the introduction of a new Cl^- -independent mechanism, such as Na^+/HCO_3^- cotransport¹³.

Because AVP stimulates both acid extruders, it could lower pH_i (see Fig. 1a) only if an acid-loading mechanism were

Table 1 Effect of EIPA and SITS on acid extrusion rate J_{ext} after a 20 mM NH_4^+ prepulse J_{ext} ($\mu\text{M s}^{-1}$)

	Control	EIPA	SITS	EIPA-sensitive (Na^+/H^+ exchange)	SITS-sensitive (HCO_3^- transport)	Acid-loading
- AVP	183 $\pm$ 4	121 $\pm$ 2	62 $\pm$ 2	62	121	0
+ AVP	326 $\pm$ 12	205 $\pm$ 10	90 $\pm$ 3	121	236	31
AVP-dep.	142 $\pm$ 8			59	115	31
% Stimulation	78			95	95	

Fluxes were calculated as the product of $d(\text{pH}_i)/dt$ and β_{total} ($\beta_{\text{total}} = \beta_{\text{HCO}_3^-} + \beta_{\text{non-HCO}_3^-}$). Values of $d(\text{pH}_i)/dt$ were determined at pH_i 6.6. $\beta_{\text{non-HCO}_3^-}$ 13.4 mM per pH unit in the presence and 14.6 mM per pH unit in the absence of AVP ($n=10$), was measured by briefly exposing mesangial cells to 10 mM NH_4^+ in the absence of Na^+ and HCO_3^- . $\beta_{\text{HCO}_3^-}$ was 9.1 mM per pH unit at pH_i 6.6. AVP increases the total J_{ext} by 142, instead of the expected 174 $\mu\text{M s}^{-1}$ ($59+115 \mu\text{M s}^{-1}$). This implies that AVP not only stimulates the two acid extruders, but also increases EIPA/SITS-insensitive acid loading to 31 $\mu\text{M s}^{-1}$, whereas in the absence of AVP there is no discrepancy. Thus, the growth factor substantially increases background acid-loading. SITS is 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate, DIDS is 4,4'-diisothiocyanostilbene-2,2'-disulphonate.

stimulated even more. To determine whether AVP enhances Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange, we determined the $\text{Cl}^-/\text{HCO}_3^-$ exchange rate from the pH_i increase induced by acute removal of external Cl^- (ref. 11). As shown in Fig. 2a, Cl^- removal reverses the exchanger, producing an HCO_3^- influx that raises pH_i . This alkalization is DIDS-sensitive (see Table 1 legend), and is larger and faster in the presence of AVP. AVP increased Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange by 129%, increasing the DIDS-sensitive J_{ext} from 313 to 717 $\mu\text{M s}^{-1}$ (Table 2). The Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchanger, which is relatively inactive at pH_i values at or above normal, does not contribute to the pH_i increase elicited by Cl^- removal. In the absence of Na^+ , AVP increased the DIDS-sensitive alkalization induced by Cl^- removal by 112% ($n=14$), close to the stimulation observed in the presence of Na^+ (data not shown).

Table 2 Effect of DIDS on equivalent net influx of HCO_3^- (J_{ext}) induced by Cl^- removal

	J_{ext} ($\mu\text{M s}^{-1}$)		
	Control	DIDS	DIDS-sensitive (HCO_3^- transport)
- AVP	333 $\pm$ 30	20 $\pm$ 1	313 $\pm$ 20
+ AVP	780 $\pm$ 40	63 $\pm$ 3	717 $\pm$ 40
AVP-dep.	447 $\pm$ 3	43 $\pm$ 4	404 $\pm$ 13
% Stimulation	134 $\pm$ 14		129 $\pm$ 17

Fluxes calculated as in Table 1, but at pH_i 7.5. $\beta_{\text{HCO}_3^-}$ was 72.5 mM per pH unit at pH_i 7.5.

An alternative assay for Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange is to acutely load the cells with alkali and monitor the rate at which pH_i returns to normal. We alkali-loaded the cells by applying and then withdrawing a solution buffered to pH 7.4 with 10% $\text{CO}_2/50 \text{ mM HCO}_3^-$. The introduction of this solution caused a sustained pH_i increase (Fig. 2b); this alkalization was absent in cells pretreated with SITS (data not shown). When the mesangial cells were returned to 5% $\text{CO}_2/25 \text{ mM HCO}_3^-$, pH_i rapidly increased because of CO_2 efflux, representing an acute alkali load. The subsequent recovery to the original pH_i is probably due to Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange, in that the equivalent HCO_3^- efflux (J_{load}) is blocked by more than 90% by either DIDS or Cl^- removal (Fig. 2b and Table 3). The Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchanger does not contribute to this pH_i decline: this transporter is normally an acid extruder, and it is virtually inactive at high pH_i ¹⁴. AVP strongly stimulates the DIDS-sensitive/ Cl^- -dependent pH_i recovery from an alkali load (Fig. 2c). The DIDS-sensitive component of J_{load} (Table 3) is increased from 568 to 1,385 $\mu\text{M s}^{-1}$, a stimulation of 143%. The DIDS-insensitive and Cl^- -independent components of the pH_i recovery are not significantly affected by AVP. Thus, $\text{Cl}^-/\text{HCO}_3^-$ exchange is stimulated by

more than 100% by AVP. Because AVP produces a fall of steady-state pH_i in control conditions, its effect in the physiological pH_i range must be to increase the amount of acid-loading by Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange more than the amount of acid extrusion by the other two transporters.

The growth factor-induced increase in pH_i is not observed in the presence of HCO_3^- despite suggestions that it may be a

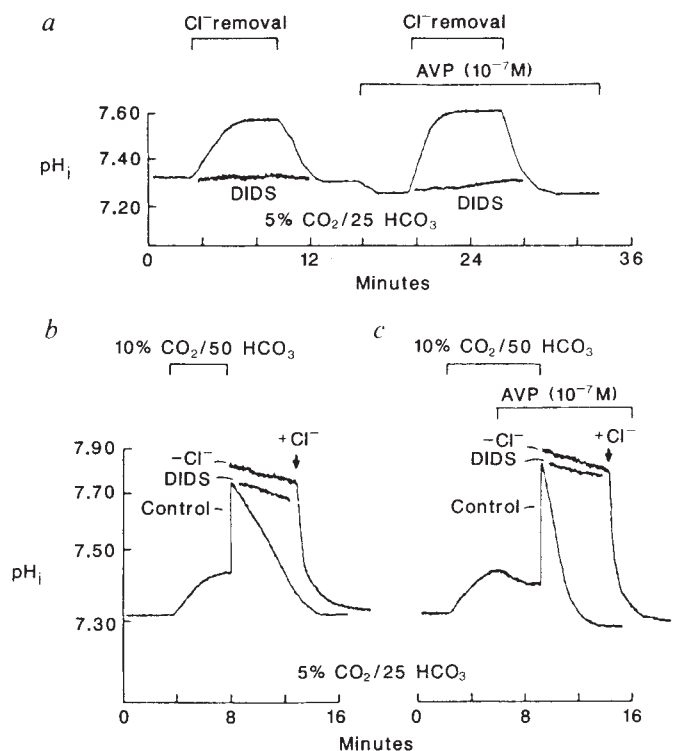


Fig. 2 Effect of 10^{-7} M AVP on Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange. **a**, $\text{Cl}^-/\text{HCO}_3^-$ exchange assessed by Cl^- removal (Cl^- replaced by aspartate) and readdition ($n=16$). Separate experiments were performed in 50 μM DIDS, which does not interfere with BCECF fluorescence ($n=16$). **b**, $\text{Cl}^-/\text{HCO}_3^-$ exchange assessed by pH_i recovery from acute alkali load in the absence of AVP. The alkali load was imposed by applying and withdrawing 10% $\text{CO}_2/50 \text{ mM HCO}_3^-$ (standard HCO_3^- solution, with 25 mM extra HCO_3^- replacing 25 mM Cl^-). The 10% $\text{CO}_2/50 \text{ mM HCO}_3^-$ solution caused pH_i to increase by 0.12 ± 0.03 ($P < 0.01$). The experiment was performed under control conditions ($n=12$), in the presence of 50 μM DIDS ($n=12$), and with Cl^- replaced with aspartate ($n=12$). **c**, $\text{Cl}^-/\text{HCO}_3^-$ exchange assessed by pH_i recovery from acute alkali load in the presence of AVP. As in (**b**), the experiment was performed under control conditions ($n=12$), in the presence of 50 μM DIDS ($n=12$), and in the absence of Cl^- ($n=12$).

Table 3 Effect of DIDS and chloride absence on acid-loading rate (J_{load}) following a 10% CO_2 prepulse

	J_{load} ($\mu M s^{-1}$)			DIDS-sensitive (HCO_3^- transport)
	Control	DIDS	No chloride	
-AVP	630 $\pm$ 22	62 $\pm$ 3	51 $\pm$ 5	568
+AVP	1,504 $\pm$ 76	119 $\pm$ 9	80 $\pm$ 3	1,385
AVP dep.	874 $\pm$ 35	57 $\pm$ 8	31 $\pm$ 7	717
% Stimulation	140 $\pm$ 11			143

Fluxes calculated as in Table 1, but at pH 7.7. $\beta_{HCO_3^-}$ was 114.9 mM per pH unit at pH_i 7.7.

crucial mitogenic signal. It could be advantageous for an activated cell to maintain a steady-state pH_i close to the unactivated level given the sensitivity to pH of nearly all cellular processes. Such a steady-state pH_i is achieved when processes that acid-load the cell (for example Na^+ -independent Cl^-/HCO_3^- exchange) balance those that alkali-load it (for example, Na^+/H^+ and Na^+ -dependent Cl^-/HCO_3^- exchange). It would also be advantageous however, for the activated cell to be able to recover more rapidly from acid and alkali loads. The requirements for both a near-normal steady-state pH_i and enhanced pH_i regulation can only be met if increases in acid loading and acid extrusion by the growth factor are comparable. This is precisely the response of the mesangial cell to AVP: application

of the growth factor causes only a small decrease in steady-state pH_i, but enormous increases in the rate at which the cell recovers from both acid (Fig. 1a) and alkali loads (Fig. 2c). This effect of AVP on pH_i does not seem to be an isolated event, as we have found eight other growth factors that also produce only a small pH_i decrease in the presence of HCO_3^- (data not shown). Thus, an important physiological effect of the growth factor is to maintain a near-normal pH_i while stimulating several acid-base transporters.

Received 12 December; accepted 28 December 1988.

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A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes

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Peptides that are antigenic for T lymphocytes are ligands for two receptors, the class I or II glycoproteins that are encoded by genes in the major histocompatibility complex, and the idiotype α/β chain T-cell antigen receptor¹⁻⁹. That a peptide must bind to an MHC molecule to interact with a T-cell antigen receptor is the molecular basis of the MHC restriction of antigen-recognition by T lymphocytes^{10,11}. In such a trimolecular interaction the amino-acid sequence of the peptide must specify the contact with both receptors: agretope residues bind to the MHC receptor and epitope residues bind to the T-cell antigen receptor^{12,13}. From a compilation of known antigenic peptides, two algorithms have been proposed to predict antigenic sites in proteins. One algorithm uses linear motifs in the sequence¹⁴, whereas the other considers peptide conformation and predicts antigenicity for amphipathic α -helices^{15,16}. We report here that a systematic delimitation of an antigenic site precisely identifies a predicted pentapeptide motif as the minimal antigenic determinant presented by a class I MHC molecule and recognized by a cytolytic T lymphocyte clone.

Synthetic peptides have been derived from the amino-acid sequence of pp89, an immediate-early (IE) phase regulatory protein of murine cytomegalovirus¹⁷⁻²⁰. The 19-mer P(161-179) contained within its 595 residues¹⁸ is an antigenic sequence²¹. This sequence H_2N -¹⁶¹GRLMYDMYPHFMPNLTG¹⁷⁹-

Table 1 Delimitation of the antigenic motif for CTL clone IE1

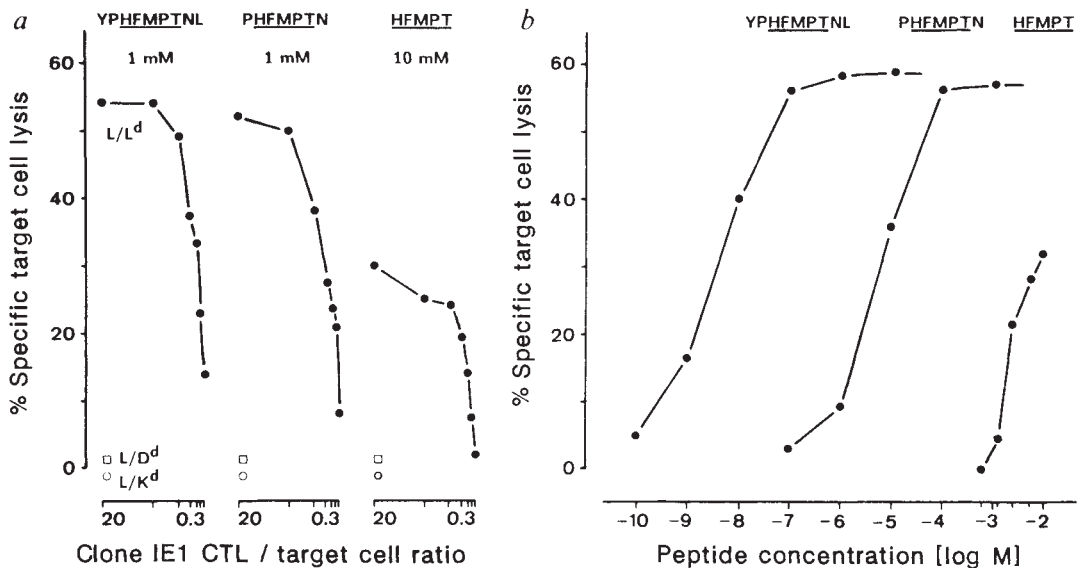
Peptide	Recognition (competition)	Peptide concentration [log M]	
		detection limit	detection saturation
11-mer: MYPHFMPNLTG	+	-7 to -6	-4 to -3
10-mers: MYPHFMPNLT	+	-9 to -8	-7 to -6
YPHFMPNLTG	+	-8 to -7	-6 to -5
9-mers: MYPHFMPN	-(-)		
YPHFMPNLT	+	-12 to -10	-9 to -7
PHFMPNLTG	+(+)	-4 to -3	-2
8-mers: YPHFMPN	-(-)		
PHFMPNLT	+(+)	-4 to -3	
7-mers: YPHFMPT	-		
PHFMPN	+	-8 to -7	-4
HFMPTNLT	+	-5 to -4	
6-mers: YPHFMP	-		
PHFMPN	+	-2 to -3	
HFMPTN	+	-2	
FMPTNLT	-		
5-mer: HFMPT	+	-3	
4-mers: HFMP	-		
FMPT	-		

Dose-response titrations of peptides give the peptide molarities in solution required for detectable target formation (detection limit) and for optimal target formation (detection saturation). The concentration ranges are compiled from at least 3 and up to 12 independent experiments.

COOH (one-letter code) contains the predicted motif HFMPT¹⁴. By screening a series of related peptides that had been reduced in length from both terminals (not all shown), we found that the nonapeptide YPHFMPNLT represents the optimal antigenic peptide (Fig. 1 and Table 1) for the cytolytic T lymphocyte (CTL) IE1 clone^{22,23}. Even though binding of peptides to class I molecules has not yet been demonstrated directly, the selective recognition on L/L^d cells implies that the L^d molecule is the

Fig. 1 Cytolytic assays demonstrating L^d -restricted recognition of peptides by CTL clone IE1 (a), and dose-response titrations of peptides (b). Each value in the peptide titrations represents the lysis determined from the plateau of a complete effector-to-target titration graph at a ratio of 20, and each value of % specific lysis is the mean of four replicate determinations.

Methods. Cytolytic effector cells: The murine (BALB/c strain; MHC^d) CTL line IE1.13-IL was derived from clone IE1 by recloning^{22,23}. Clone IE1 expresses an α/β (V β 6) TCR/CD3 complex and displays the surface phenotype CD4⁻CD5⁻CD8⁺Thy-1⁺. L fibroblast transfectants: L/L^d cells were derived from L cells by transfection with the L^d gene, and L/D^d and L/K^d transfectants were provided by M. Cochet and J. P. Abastado (Institut Pasteur). Surface expression of the L^d, D^d and K^d molecules was confirmed by cytofluorography with monoclonal antibodies. Synthetic peptides: Peptides were synthesized by using the Barany-Merrifield solid-phase technique on an Applied Biosystems Peptide Synthesizer, purified preparatively and analysed as described previously¹³. Preparation of target cells and cytolytic assay: Target cells were labelled at 37 °C for 1 h with Na₂[⁵¹Cr]O₄. The radioactive cells were then distributed in aliquots of 10⁵ cells and incubated in 0.2 ml for a further 1 h at 27 °C with peptides at defined molarities (1 M corresponds to 10¹⁵ peptide molecules per cell) dissolved in RPMI 1640 culture medium supplemented with 2% of FCS. The highest peptide concentration tested was 10⁻² M. This limit was imposed by the solubility of the peptides. After washing to remove excess peptide and ⁵¹Cr, a standard 3-h cytolytic assay was performed at 37 °C with 1,000 target cells and graded numbers of clone IE1 CTL. Competition assays: The ability of peptides to compete with the optimal antigenic nonapeptide YPHFMPTNL was tested in two ways. Either the concentration of the antigenic peptide was kept constant at 10⁻⁷ M and the competitor peptide titrated up to 10⁻⁴ M, or the concentration of the competitor peptide was kept constant at 10⁻⁴ M and the antigenic peptide was titrated from 10⁻⁵ to 10⁻¹⁰ M. In both cases the L/L^d target cells were pre-incubated for 30 min with the competitor peptide before the antigenic peptide was added.



receptor involved in the interaction with the T-cell antigen receptor (TCR) of clone IE1 for the family of peptides tested (Fig. 1a). Titration of the nonapeptide YPHFMPTNL, the heptapeptide PHFMPTN, and the pentapeptide HFMPPT resulted in dose-response saturation curves showing thousandfold differences in antigenic potency (Fig. 1b). The data refer to a one-hour incubation of target cells with peptide. Differences in antigenic potency are also reflected by the time needed for optimal target formation; five minutes is long enough to prepare an optimal nonapeptide target, which is in line with data for a high-affinity peptide²⁴, whereas plateau lysis could be achieved with the pentapeptide only when the peptide pulse was prolonged to two hours or more (not shown). Because clone IE1 does not discriminate between nonapeptide and pentapeptide target once the target is formed, the limiting step is apparently the association between the peptide ligand and its MHC receptor on the target-cell surface. We therefore conclude that HFMPPT forms the antigenic core of the peptides by comprising both the complete epitope for the TCR of clone IE1 and an agretope that is adequate, but not optimal for specification of the interaction with L^d.

The deletion of Tyr1 (Y) from peptide YPHFMPTNL diminished and the deletion of Leu 9 (L) destroyed the antigenicity, and the resulting octapeptides PHFMPTNL and YPHFMPTN both also failed to compete with YPHFMPTNL (Table 1). According to the currently used theorem for classifying residues as T-cell contact residues or as MHC-molecule contact residues¹², Tyr1 and Leu 9 would have been interpreted as residues contacting the MHC molecule. This conclusion, however, was proven incorrect by the findings that PHFMPTN, which lacks both residues, was >10³-fold more antigenic than PHFMPTNL (Table 1) and could not be competed by a 10³-fold excess of YPHFMPTN (not shown), although both octapeptides include the heptapeptide sequence entirely. Results compiled

in Table 1 follow a consistent pattern from the pentameric core motif up to the nonapeptide size, in that symmetric additions of residues improved the antigenicity, whereas addition of residues at either end had a negative effect.

This communication adds new aspects to the current understanding of the MHC molecule-peptide-TCR interaction. The first aspect concerns the minimal length of antigenic peptides. Until now, the shortest peptides reported as antigenic were heptapeptides presented by MHC class II molecules^{25,26}. A recent study described a nonapeptide presented by an MHC class I molecule as minimal antigenic peptide of the glycoprotein of LCMV²⁷. It should be emphasized that in our example systematic shortening from both termini was critical for the identification of the pentapeptide as the minimal antigenic peptide. Since the sequence of this pentapeptide is a predicted pentameric motif, and as most motifs are tetrameric¹⁴, recognition even of tetrapeptides may be possible.

The second aspect concerns the classification of epitope and agretope residues. For the hen egg-white lysozyme peptide (52-61), Allen *et al.*¹² defined residues as MHC-antigen contact residues when a substitution led to the loss of both antigenicity and the ability to compete with the unmodified antigenic peptide. With this approach and proposing a helical conformation, agretope residues were segregated from epitope residues. From a set of overlapping peptides, Sette *et al.*²⁸ predicted a heptameric core in a planar conformation for the ovalbumin peptide (323-339). In contrast to these examples, we have positively identified the motif HFMPPT as an antigenic core in pp89 peptides by demonstrating a direct antigenicity of the pentapeptide HFMPPT. The important implication is that residues whose deletion causes loss of both antigenic potency and competitive ability are not necessarily agretope residues in the classical sense of MHC-receptor binding sites. We propose that residues flanking an antigenic core motif can affect the antigenic potency positively

or negatively by their influence on peptide conformation.

We thank Drs U. Weber and H. Kalbacher of the Physiologisch-chemisches Institut der Universität Tübingen for preparation of some of the peptides, and Dr Margarita Del Val for critical reading of the manuscript. The technical assistance of Irene Huber and the secretarial help of Sabine Grau is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft.

Received 23 September 1988; accepted 3 January 1989.

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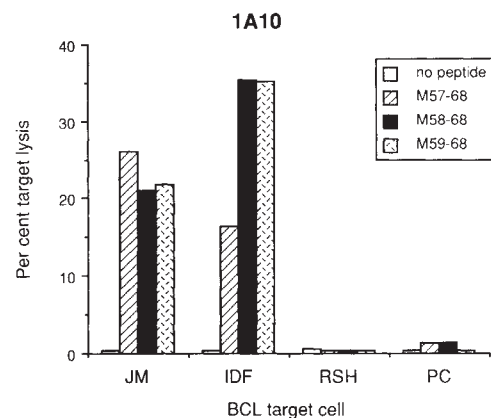


Fig. 1 CTL are cross-restricted, recognizing peptide on both autologous and Aw69-expressing target cells. Recognition by CTL clone 1A10 from the donor JM (A2, 2; B15, 51; DR4, 4) of peptides M57-58, M58-68, and M59-68 of influenza matrix protein on autologous BCL target cells (JM), three HLA-mismatched BCL, IDF (A26, w69; B18, 38; DR11, 11), RSH (A68, 68; B42, 42; DR3, 3) and PC (A3, 24; B37, 62; DR6, 6). $K/T = 1.5$, peptides present throughout assay $10 \mu\text{M}$.

Methods. CTL clone 1A10 was derived from a parent line from the donor, JM. Peripheral blood lymphocytes were separated from whole blood, stimulated initially with A/X31 virus (grown and stored as previously described¹⁸) and maintained in culture by repeated stimulations with peptide M57-68, as previously described⁴. CTL were cloned by selection of CD8 positive cells (labelled with B941 antibody from Dr C. Mawas, and fluorescein-labelled anti-mouse immunoglobulin) using an Ortho Cytofluorograf cell sorter, into 96-well plates containing 10^4 irradiated BCL (pretreated with $50 \mu\text{M}$ M57-68) and IL-2 10 U ml^{-1} (Cetus Corp.) in RPMI containing 10% fetal calf serum. Monoclonality was indicated by the presence of a single β -chain gene rearrangement on Southern blotting (data not shown). Cytotoxic activity was assessed by incubating EBV-transformed lymphoblastoid cells (BCL), labelled with ^{51}Cr , with CTL and diluted peptide (where appropriate) for 4 h in round-bottomed 96-well plates. Per cent target lysis was calculated from the formula $(E - M/D - M) \times 100$ where E = experimental ^{51}Cr release, M = release in presence of culture medium (always $<18\%$) and D = release by 5% Triton X-100. For details of the peptides see legend to Fig. 3.

Class I cross-restricted T cells reveal low responder allele due to processing of viral antigen

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Cytotoxic T lymphocytes (CTL) recognize protein antigens which have been processed by the target cell and then presented in association with the relevant class I molecule of the major histocompatibility complex (MHC)¹. Short synthetic peptides, which are able to associate directly with target cells¹, may substitute for these processed fragments in stimulating antigen-specific CTL responses. Using this approach, a dominant HLA-A2-restricted epitope has previously been mapped to residues 58-68 of influenza A virus matrix protein². Here we report HLA-A2-restricted CTL which are also able to recognize this short synthetic peptide in association with HLA-Aw69, but which fail to recognize HLA-Aw69 expressing cells infected with influenza A virus. Furthermore, individuals possessing HLA-Aw69 who respond to influenza A virus, do not respond to M58-68. These results imply that the low response to this epitope on infection of HLA-Aw69 individuals with influenza A is due to failure of the naturally processed product of matrix protein to associate with Aw69.

Individuals who express the common HLA-A2 variant, HLA-A2.1 (HLA-A*0202 as defined in ref. 3), and who can mount a cytotoxic response to influenza infected target cells, following *in vitro* stimulation with virus have been shown, in all cases tested to date, to possess CTL that specifically recognize synthetic peptides representing residues 56-68 of the matrix protein⁴. Individuals who have other variants of A2, or the closely

related allele HLA-Aw69, which differs by six amino acids from A2*0202 (listed in Table 1)⁵, did not respond to this region of matrix protein⁴.

We made several CTL lines and clones from a single HLA-A2 expressing individual, JM, some of which were able to recognize matrix peptide 56-68 in association with HLA-Aw69 as well as A2, although they were otherwise HLA-restricted (Fig. 2). CTL clone 1A10 was able to recognize the short synthetic peptides M57-68, M58-68 and M59-68 in association with both the autologous (JM) HLA-A2-expressing EBV-transformed B lymphoblastoid cell line (BCL), and a BCL expressing Aw69 which did not share any HLA alleles with JM (Fig. 1), but did not recognize two other HLA mismatched BCL in the presence of these peptides.

Although these matrix-specific A2-restricted CTL were able to recognize Aw69-expressing target cells in the presence of synthetic peptide, a discrepancy between recognition of A2 and Aw69 BCL was seen following A/X31 virus infection of the target cells (Fig. 2). Both autologous and A2-matched BCL were recognized following pretreatment with the peptide, or infection with influenza virus (Fig. 2a, e). In contrast Aw69-expressing BCL were very poorly recognized following virus infection, although they were well recognized after peptide treatment (Fig. 2b, c, f). In addition, virus-specific polyclonal CTL from an Aw69-expressing donor were unable to recognize the synthetic peptide M58-68 (Fig. 2d). Even with repeated stimulation of these latter CTL with M58-68 *in vitro*, we were unable to obtain a response from either of two Aw69-expressing (HLA identical)

Fig. 2 HLA-Aw69-expressing BCL can present peptide, but not virus to A2-restricted matrix specific CTL, whereas an Aw69-expressing donor is able to respond to virus, but not matrix peptide. Polyclonal CTL line JM-16 was assayed for recognition of autologous BCL (a), or Aw69-expressing BCL LLICRF (A1, w69; B8, 35; DR10, 17) (b) or WI (Aw68, w69; B22, 51; DR2, 13) (c); target cells (10^4 per well), were either untreated (open circles), pretreated with 50 μ M M58-68 (closed circles) or infected with A/X31 (closed triangles). Polyclonal CTL from donor HI (HLA-identical brother to WI) were assayed for recognition of WI target cells (d). In a separate experiment, the A2-restricted CTL clone, 1A10 (derived as in Fig. 1), was assayed for recognition of a BCL matched only through A2, (SC: A2, 2; B27, 27; DR2, 2) (e), or the Aw69-expressing BCL, WI (f).

Methods. JM-16 was derived as described in the legend to Fig. 1. Polyclonal CTL from donor HI were prepared by stimulating peripheral blood lymphocytes with A/X31 virus in culture for 7 days, as previously described⁴. The 51 Cr-release assay, was performed as for Fig. 1 except that BCL target cells were preincubated for 1 h with either medium, peptide or A/X31 virus, washed, and then rested in 1 ml medium at 37 $^{\circ}$ C for 3 h before 51 Cr-labelling, and addition to the assay.

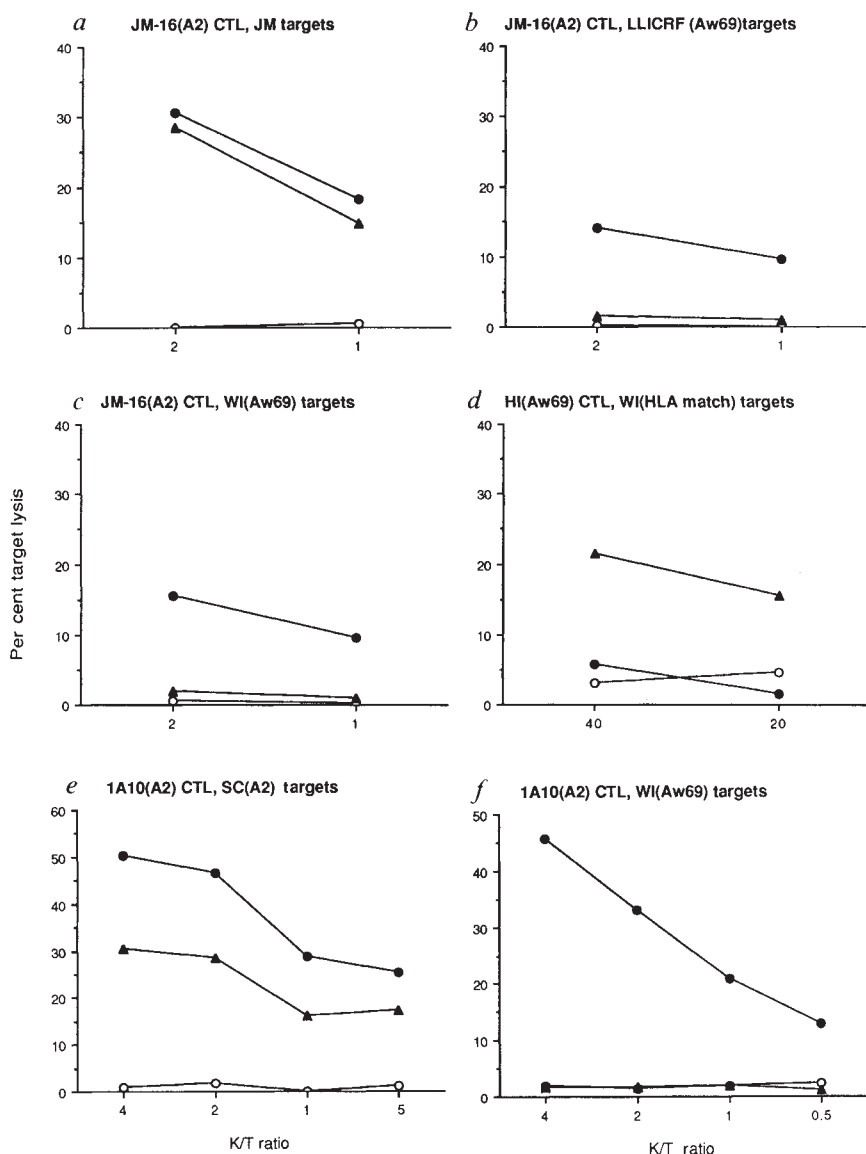
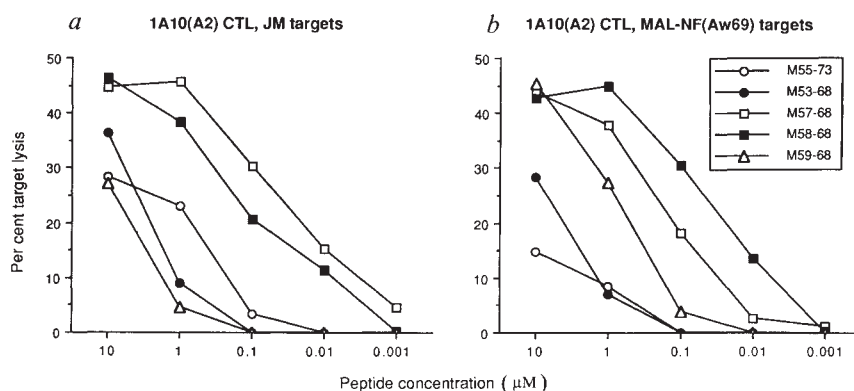


Fig. 3 The pattern of peptide recognition varies depending on whether the target cell expresses A2 or Aw69. CTL clone 1A10 ($K/T = 2$) was assayed for recognition of peptides of varying lengths in association with autologous BCL (a) or a BCL expressing Aw69 (MAL-NF: A1, w69; B35, 57; DR10, 17) (b) using 10^4 target cells per well. Non-specific lysis, that is in the absence of peptides, was 0% for both targets.

Methods. Influenza A virus matrix peptides M55-73 (leu-thr-lys-gly-ile-leu-gly-phe-val-phe-thr-leu-thr-val-pro-ser-glu-arg-gly); M53-68 (ser-pro-...) M55-68, M57-68, M58-68 and M59-68 were synthesized and donated by Dr J. Rothbard. The 51 Cr-release assay was performed as for Fig. 1.



donors. Thus, although Aw69 was able to present the synthetic peptide to CTL, Aw69 donors were non-responders to this epitope.

It is possible that patterns of immunodominance in a T-cell response may be influenced by other MHC molecules⁵ or by other background genes⁶. But Aw69 target cells with different MHC types were all poor presenters to the A2-restricted CTL following virus infection (an example is shown in Fig. 2b).

Therefore we propose that the Aw69 molecule itself, although binding the synthetic peptide well, may associate poorly with a naturally produced fragment. Peptide M58-68 is the optimum length for maximal target lysis by matrix-specific CTL restricted through HLA-A2 and cross-restricted through Aw69. There is, however, no evidence that this is the actual length of peptide produced when whole matrix protein is processed by a cell. We therefore assayed for killing of A2 or Aw69 targets using a

Table 1 Amino-acid differences between the sequences of HLA-A*0202 and HLA-Aw69

Position	A*0202	Aw69	Location†
9	Phe	Tyr	β -sheet, in
62	Gly	Arg	α -helix, up
63	Glu	Asn	α -helix, in
66	Lys	Asn	α -helix, in
70	His	Gln	α -helix, in
74	His	Asp	α -helix, in

† All amino-acid differences are in the α_1 domain, orientation is indicated, either pointing into the putative peptide binding site (in), or pointing towards the T-cell receptor (up), according to the crystal structure determined by Bjorkman *et al.*^{8,9}.

number of peptides of varying lengths from this region (Fig. 3). The dose response curves of recognition by the CTL clone 1A10 of peptide M58–68 were very similar for both A2- (autologous) and Aw69-expressing target cells (Fig. 3). But recognition of the shorter peptide, M59–68 was more efficient on the Aw69 target, whereas recognition of the longer peptides, particularly M55–73, was approximately tenfold less efficient. This decreased efficiency may be sufficient to account for the low response if natural peptides are present in low concentrations.

HLA-Aw69, a rare allele, is the result of a natural exon shuffle between HLA-A2 and HLA-Aw68 (ref. 7) with the α_1 domain from Aw68 and the α_2 and α_3 domains from A2. Although there are only six amino-acid differences between Aw69 and A2 (listed in Table 1), reference to the crystal structure of A2, as defined by Bjorkman *et al.*^{8,9}, suggests that these occur in areas likely to be important for either peptide binding or direct recognition of the MHC molecule by the T-cell receptor. Cross-restriction for recognition of foreign antigen by class I-restricted T cells has been reported infrequently. Human HLA-Aw69 restricted Epstein-Barr virus-specific CTL were able to recognize Aw68 (sharing the α_1 domain), but not A2-expressing BCL¹⁰. In mice, an exchange of the exons coding for the α_1 and α_2 domains of H-2 L^d and D^d maintained epitopes recognized by CTL specific for influenza or Sendai virus but not vesicular stomatitis virus¹¹. Other reports of exon shuffles between the α_1 and α_2 domains have not identified cross-restricted CTL^{12–14}.

Cross-restricted CTL recognizing cyanogen bromide fragments of ovalbumin on target cells expressing three different H-2 types have been generated by priming *in vitro*^{15,16} but these T cells were unable to recognize target cells expressing a transfected ovalbumin gene. A similar result was found with MHC class II-restricted T-cell clones, produced by priming mice with peptide *in vitro* in that the clones responded on processing and presentation of the native molecule¹⁷. In both these cases, the T-cell responses were generated against peptides that were not epitopes when the animals were primed with the native proteins. Here, we show that HLA-Aw69 can present a synthetic peptide to A2-restricted CTL, induced by natural infection, even though Aw69 is a non-responder allele to the native virus antigen. All of these results imply that the processing system limits the complexity of naturally induced T-cell immune responses.

We thank Dr N. Carter for operating the cell sorter, Dr A. Ting for HLA typing, Dr J. Rothbard for the peptides, Dr H. Festenstein for the cell line IDF, CETUS Corporation for recombinant IL-2 and Dr J. Bodmer for the cell lines LLICRF and MAL-NF. H.C.B. is in receipt of a Medical Research Council training fellowship. This work was supported by the MRC.

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Characterization of the yeast *HSP60* gene coding for a mitochondrial assembly factor

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The hsp60 protein isolated from the protozoan *Tetrahymena thermophila* is induced in response to heat stress and is a member of an immunologically conserved family represented in *Escherichia coli* and in mitochondria of plants and animals^{1,2}. We report here the cloning and characterization of a nuclear gene, *HSP60*, which codes for the hsp60 homologue from the yeast *Saccharomyces cerevisiae*. Nucleotide sequence analysis revealed that yeast hsp60 is related to the groEL protein of *E. coli*^{3–6} and the RUBISCO-binding protein (RBP) of chloroplasts⁷. *HSP60* was found to be the genetic locus of the conditional-lethal mutation described by Cheng *et al.*⁸, which at non-permissive temperature is defective in the assembly of several different multisubunit complexes in mitochondria. These data are consistent with the hypothesis that the groEL-related proteins serve an evolutionarily conserved function as accessory factors facilitating the folding and/or association of individual subunits of multimeric protein complexes^{7,9–14}.

Polyclonal antiserum raised against purified *T. thermophila* hsp60 (ref. 1) was used to isolate a segment of the yeast *HSP60* gene by screening an expression library of genomic DNA fragments ligated into the λ -phage vector λ gt11 (ref. 15). Analysis of a 3.0 kilobase (kb) yeast DNA fragment present in one positive clone revealed an open reading frame coding for a protein homologous to both groEL and RBP. We used this DNA as a hybridization probe to isolate an overlapping segment of the yeast genome which contained the entire *HSP60* coding sequence (Fig. 1).

The sequence of a 1.8-kb region containing *HSP60* is presented in Fig. 3, along with the derived amino-acid sequence. The *HSP60* gene codes for a polypeptide of 572 amino acids (relative molecular mass (M_r) 60,830) which is similar to both groEL and RBP (Fig. 4). Based on our previous finding that the hsp60 family of proteins is structurally and immunologically similar to groEL², we have defined this open reading frame as the yeast *HSP60* gene. Northern hybridization analyses showed that *HSP60* is transcribed into a mRNA of ~1.9 kb, commensurate with the estimated 64,000 (64K) M_r of hsp60 (Fig. 2). This mRNA is induced 2–3-fold above basal levels when cells are heat-stressed, in agreement with the heat-shock inducibility of hsp60 (refs 1, 2) (Fig. 2). Increasing the copy number of *HSP60* by transformation with the multicopy plasmid YEpHSP60 (Fig. 1) results in higher concentration of immunoreactive material in mitochondria (Fig. 2), providing further evidence for the identity of this gene.

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Received 7 December 1988; accepted 10 January 1989.

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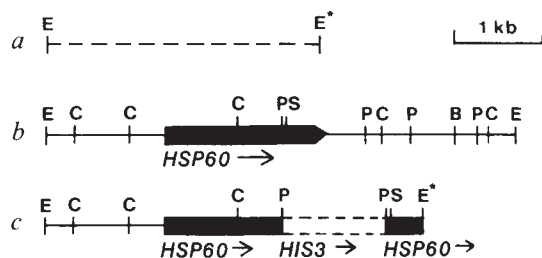


Fig. 1 Restriction maps of *HSP60* and *hsp60::HIS3*. *a*, The dotted line indicates the region of yeast genomic DNA isolated from a λ gt11 clone which reacts with polyclonal antiserum raised against *hsp60* purified from *Tetrahymena*¹. The *EcoRI* site designated *E** was inserted as a linker during construction of the library. *b*, restriction map of the genomic DNA fragment containing the region depicted in *a*. The black bar indicates the coding region of *HSP60* as determined by nucleotide sequence analysis, with the direction of transcription indicated by the arrow. Plasmid YEpHSP60 is comprised of this 5.3-kb *EcoRI* fragment cloned in the 2 μ circle-based shuttle vector YEp352 (ref. 20). *c*, Restriction map of the null allele *hsp60::HIS3*. Black bars indicate the coding regions of *HSP60*, and dotted lines indicate a *HIS3*-containing fragment which interrupts *HSP60* by insertion at the *PstI* site. The positions of restriction enzyme recognition sites are indicated for *EcoRI* (E), *BclI* (C), *PstI* (P), *SalI* (S) and *BamHI* (B).

Methods. A library of yeast genomic DNA fragments in λ gt11 (ref. 15) was screened with a polyclonal antiserum reactive with *hsp60* (ref. 1). From 300,000 library recombinants screened, a single positive plaque was isolated which upon repurification gave only positive clones. The positive recombinant contained three *EcoRI* fragments within the λ arms, one of which hybridized to a heat-shock-inducible mRNA. This 3.0-kb fragment is depicted in *a*. Nucleotide sequence analysis of the ends of the 3.0-kb fragment revealed an open reading frame homologous to *groEL* which continued through the site marked *E** in the 5'-3' direction. Therefore, we used the 3.0-kb fragment as a hybridization probe to isolate an overlapping genomic fragment containing the entire *HSP60* gene. Southern hybridization analysis showed that the 3.0-kb fragment isolated from λ gt11 was derived from a genomic *EcoRI* fragment of ~5 kb. A library of *EcoRI* fragments with a size of ~5 kb was prepared in the 'phagemid' vector pUC118 (ref. 21) and screened with the 3.0-kb fragment. Positive clones contained the 5.3-kb *EcoRI* fragment shown in *b*. Nucleotide sequence analysis (Fig. 3) revealed the location of *HSP60*. To construct *hsp60::HIS3* the 3.0-kb *EcoRI* fragment in *a* was cloned into the plasmid vector YEp352E, a derivative of YEp352 from which the *PvuII* fragment containing the partial *lac* operon and multiple cloning site was removed and replaced with an *EcoRI* linker. The recombinant plasmid was linearized at the unique *PstI* site within *HSP60* and ligated to a 1.15-kb fragment of yeast DNA containing the wild-type *HIS3* gene²². One of the *PstI* sites delimiting this fragment is located downstream of the *HIS3* coding region²², whereas the other *PstI* site is derived from the pUC18 multiple cloning site. The linear *EcoRI* fragment shown in *c* was excised from YEp352E and used to transform the *his3⁻/his3⁻* diploid *a/aW303* to histidine independence²³. Southern hybridization analysis of chromosomal DNA verified that the *His⁺* transformants had integrated the disrupted allele at the homologous site in one chromosome, and were of the genetic constitution *HSP60/hsp60::HIS3* (data not shown).

Comparison of the *HSP60* and *groEL* products reveals 54 per cent amino-acid identity, given five small insertion/deletions, whereas comparison with RBP reveals 43 per cent identity including four insertion/deletions (Fig. 4). Regions of similarity are distributed evenly throughout the lengths of the proteins; one exception occurs at the carboxyl terminus, where the repeated Gly-Gly-Met motif is conserved in both *hsp60* and *groEL*, but not in RBP. *Hsp60* contains an amino-terminal extension of ~22 amino acids which is not represented in two other members of this gene family. This sequence is characteristic of most mitochondrial targeting peptides¹⁶ in that it contains

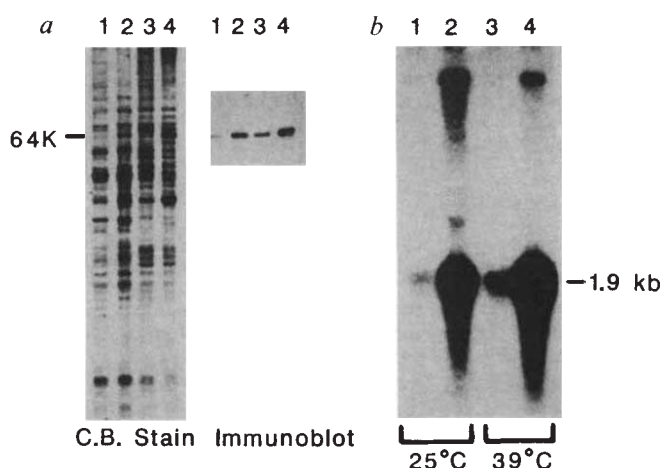


Fig. 2 *a*, Immunoblot analysis of cells containing single or multiple copies of *HSP60*. Haploid strain *aW303* (lanes 1, 3) or the same strain transformed with YEpHSP60 (lanes 2, 4) were grown to mid-log phase at 30°C. Sixty micrograms of total protein extracts²⁶ (lanes 1, 2) or purified mitochondria²⁷ (lanes 3, 4) were run in duplicate SDS-polyacrylamide gels. Proteins in one gel were stained with Coomassie blue, whereas those in the second gel were transferred to nitrocellulose paper and probed with *hsp60*-reactive antiserum as previously described¹. Raising the copy number of *HSP60* by including the gene on a multi-copy plasmid increases the cellular level of *hsp60*-specific immunoreactive protein. Relative molecular mass standards included on the stained gel (not shown) showed the immunoreactive material to be of *M_r* 64K, in agreement with our previous analyses^{1,2}. Thus, there is a discrepancy between the apparent *M_r* of *hsp60* determined by polyacrylamide gel electrophoresis and the value predicted by the nucleotide sequence of the gene (61 K including the putative mitochondrial targeting peptide). *b*, Steady state levels of *HSP60* mRNA in normal and heat-stressed conditions. Strain *aW303* (lanes 1, 3) or the same strain transformed with YEpHSP60 (lanes 2, 4) were grown to mid-log phase at 25°C. The cultures were then divided and grown for a further 2 hours either at 25°C (lanes 1, 2) or 39°C (lanes 3, 4). Total nucleic acids were extracted²⁸, denatured with glyoxal²⁹, and separated by agarose gel electrophoresis. Nucleic acids were transferred to nitrocellulose filters and analysed by Northern blotting, using the 3.0-kb fragment shown in Fig. 1*a* as a radiolabelled probe. The probe hybridizes to a single RNA band of 1.9 kb (RNA relative-molecular mass standards not shown). The concentration of this mRNA is two to three times higher in the heat-shocked cells than in the non-stressed control. Raising the copy number *HSP60* increases the steady-state level of the 1.9-kb mRNA at 25°C, and heat-shock induction is again observed. The high *M_r* band seen in lanes 2 and 4 is due to hybridization of the probe to DNA.

five basic and no acidic residues, and contains a total of six hydroxylated residues. The presence of this targeting sequence is consistent with the mitochondrial location of *hsp60* (refs 1, 2).

One-step gene disruption¹⁷ was used to construct an allele of *HSP60* which is inactive because of insertion of the *HIS3* gene (Fig. 1). The diploid strain *a/aW303VHSP60* was constructed with one wild-type allele and one null allele designated *hsp60::HIS3*. This strain is homozygous *his3⁻*, so the wild-type and null alleles of *HSP60* can be scored by histidine auxotrophy and prototrophy, respectively. Following dissection of tetrads derived from *a/aW303VHSP60*, only two of the four meiotic products from each ascus formed colonies on non-selective medium; replica plating revealed that all surviving progeny required histidine and thus had received the wild-type allele. Because no haploid cells containing *hsp60::HIS3* form colonies, we conclude that *HSP60* is essential for cell viability in yeast. Cells were grown at 30°C, thus the requirement for *HSP60* is not limited to conditions of heat stress.

5' - ^{BclI} TGATCAAGTA

-410

-400 GAAGCCCAAGACTAGTTGTGCAAGAAGTACAGTTGAAATCCAGCCAGTTATGTCTCATCCCTATTCCACTGTTTTGTAGGTATACAAATGGTGTACG

-300 AGCATTGAAGCTGTTTATTGCTGGTATTACCTCTGGAACCTACTTTATAGCGCCCATCTGTATATGTCATCATATACGGACCTGTGGAATTTTCCA

-200 GAAAACCAATGGGAGGGTGACTTTAAATTTGCTTCTTTGTCGGAGCAGATCCAGACGGACATTATGTATATATATGAAGGAGACTAAGTTTT

-100 ACATTCATTATGTAAGGTGCTGGTTCGCTTCATGCATCTCTGAATAATATAAGAAAAATCCACGAGAAAAACATACAGCAAAAAAGCTTTTCAAA

+1 ATG TTG AGA TCA TCC GTT GTT CGT AGT CGC GCT ACT TTA AGG CCT TTA TTG CGT CGT GCT TAC TCC TCT CAT AAA
Met Leu Arg Ser Ser Val Val Arg Ser Arg Ala Thr Leu Arg Pro Leu Leu Arg Arg Ala Tyr Ser Ser His Lys

+76 GAA TTG AAA TTC GGT GTA GAA GGA AGA GCC TCC CTT CTT AAG GGT GTC GAA ACT TTA GCT GAA GCG GTT GCT GCT
Glu Leu Lys Phe Gly Val Glu Gly Arg Ala Ser Leu Leu Lys Gly Val Glu Thr Leu Ala Glu Ala Val Ala Ala

+151 ACT TTG GGT CCA AAG GGT AGA AAC GTT TTA ATC GAA CAG CCT TTC GGT CCT CCA AAG ATT ACT AAG GAT GGT GTT
Thr Leu Gly Pro Lys Gly Arg Asn Val Leu Ile Glu Lys Pro Phe Gly Ser Gln Val Ala Val Glu Lys Val Glu Gly

+226 ACA GTT GCC AAA TCT ATT GTG TTG AAG GAC AAG TTT GAA AAT ATG GGT GCC AAG TTA CTA CAA GAA GTT GCC TCC
Thr Val Ala Lys Ser Ile Val Leu Lys Asp Lys Phe Glu Asn Met Gly Ala Lys Leu Leu Gln Glu Val Ala Ser

+301 AAA ACC AAT GAG GCT GCT GGT GAC GGT ACT ACT TCT GCT ACT GTT TTA GGT AGA GCC ATC TTC ACA GAA TCC GTC
Lys Thr Asn Glu Ala Ala Gly Asp Gly Thr Thr Ser Ala Thr Val Leu Gly Arg Ala Ile Phe Thr Glu Ser Val

+376 AAA AAT GTC GCC GCT GGT TGT AAC CCT ATG GAT TTG AGA AGG GGT TCT CAA GTT CCA GTT GAA AAA GTG ATT GAA
Lys Asn Val Ala Ala Gly Cys Asn Pro Met Asp Leu Arg Arg Gly Ser Gln Val Ala Val Glu Lys Val Ile Glu

+451 TTT TTG AGC GCC AAC AAG AAA GAA ATT ACC ACA TCT GAG GAA ATT GCT CAA GTA GCA ACC ATT TCT GCC AAT GGG
Phe Leu Ser Ala Asn Lys Lys Glu Ile Thr Thr Ser Glu Glu Ile Ala Gln Val Ala Thr Ile Ser Ala Asn Gly

+526 GAC TCT CAT GTT GGT AAG TTA CTA GCT TCA GCT ATG GAA AAG GTT GGA AAA GAA GGT GTC ATC ACT ATC AGA GAA
Asp Ser His Val Gly Lys Leu Leu Ala Ser Ala Met Glu Lys Val Gly Lys Glu Gly Val Ile Thr Ile Arg Glu

+601 GGT AGA ACA TTG GAA GAT GAA CTT GAG CTT ACT GAA GGT ATG AGG TTT GAT CGT GGT TTT ATT TCT CCA TAC TTC
Gly Arg Leu Lys Pro Glu Asp Glu Lys Thr Ile Glu Lys Met Arg Phe Ser Asp Arg Gly Phe Ile Ser Pro Tyr Phe

+676 ATC ACT GAT CCA AAG TCG AGC AAG GTG GAA TTT GAA AAG CCA TTG CTA TTG TTG AGT GAA AAG AAA ATT TCT TCC
Ile Thr Asp Pro Lys Ser Ser Lys Val Glu Phe Glu Lys Pro Leu Leu Leu Leu Ser Glu Lys Lys Ile Ser Ser

^{BclI}

+751 ATT CAA GAT ATC TTG CCA GCT TTG GAA ATT TCC AAT CAA AGC AGA AGA CCT TTG TTG ATC ATT GCT GAA GAT GTT
Ile Gln Asp Ile Leu Pro Ala Leu Glu Ile Ser Asn Gln Ser Arg Arg Pro Leu Leu Ile Ile Ala Glu Asp Val

+826 GAC GGT GAA GCT CTT GCG GCC TGT ATT TTG AAC AAG TTA AGG GGT CAA GTT AAG GTT TGT GCT GTG AAG GCG CCT
Asp Gly Glu Ala Leu Ala Ala Cys Ile Leu Asn Lys Leu Arg Gly Gln Val Lys Val Cys Ala Val Lys Ala Pro

+901 GGT TTG GGT GAT AAT AGA AAG AAT ACA ATT GGT GAT ATT GCA GTC TTG ACC GGC GGT ACT GTT TTT ACT GAG GAG
Gly Phe Gly Asp Asn Arg Lys Asn Thr Ile Gly Asp Ile Ala Val Leu Thr Gly Gly Thr Val Phe Thr Glu Glu

+976 TTG GAT TTG AAA CCA GAA CAA TGT ACC ATA GAA AAC TTG GGT TCT TGT GAC TCT ATT ACC GTT ACT AAG GAA GAC
Leu Asp Leu Lys Pro Glu Lys Thr Ile Glu Asn Leu Gly Ser Cys Asp Ser Ile Thr Val Thr Lys Glu Asp

+1051 ACC GTT ATC CTG AAC GGT AGT GGT CCA AAG GAA GCT ATT CAA GAG AGA ATT GAA CAA ATC AAG GGC TCC ATC GAC
Thr Val Ile Leu Asn Gly Ser Gly Pro Lys Glu Ala Ile Gln Glu Arg Ile Glu Gln Ile Lys Gly Ser Ile Asp

+1126 ATT ACC ACC ACA AAT TCA TAT GAG AAG GAG AAA CTG CAA GAG CGT TTG GCC AAA TTG TCC GGC GGT GTT GCT GTC
Ile Thr Thr Thr Asn Ser Tyr Glu Lys Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ser Gly Gly Val Ala Val

+1201 ATC AGG GTC GGT GGT GCA TCT GAA GTT GAA GTT GGT GAA AAG AAG GAC CGT TAC GAT GAT GCT TTG AAC GCT ACC
Ile Arg Val Gly Gly Ala Ser Glu Val Glu Lys Thr Ile Glu Lys Lys Asp Arg Tyr Asp Ala Leu Asn Ala Thr

+1276 AGA GCT GCA GTT GAG GAA GGT ATC TTG CCA GGT GGT GGT ACT GCC TTA GTG AAG GCA TCT AGA GTT TTG GAT GAA
Arg Ala Ala Val Glu Glu Gly Ile Leu Pro Gly Gly Gly Thr Ala Leu Val Lys Ala Ser Arg Val Leu Asp Glu

^{PstI}

+1351 GTT GTT GTC GAC AAT TTG GAT CAA AAA TTG GGT GTC GAT ATC ATA AGA AAG GCC ATT ACA AGA CCA GCC AAG CAG
Val Val Val Asp Asn Phe Asp Gln Lys Leu Gly Val Asp Ile Ile Arg Lys Ala Thr Arg Pro Ala Lys Gln

+1426 ATC ATT GAA AAC GCT GGT GAA GAA GGT TCA GTT ATC ATC GGC AAA TTG ATT GAT GAA TAT GGT GAT GAT TTT GCC
Ile Ile Glu Asn Ala Gly Glu Glu Gly Ser Val Ile Ile Gly Lys Leu Ile Asp Glu Tyr Gly Asp Asp Phe Ala

+1501 AAG GGT TAC GAT GCC TCT AAG TCA GAA TAC ACC GAC ATG TTA GCC ACT GGT ATC ATC GAT CCA TTT AAA GTG GTT
Lys Gly Tyr Asp Ala Ser Lys Ser Glu Tyr Thr Asp Met Leu Ala Thr Gly Ile Ile Asp Pro Phe Lys Val Val

+1576 AGA TCC GGT TTA GTT GAT GCT TCT GGT GTT GCC TCA CTA TTA GCT ACT ACC GAA GTT GCT ATT GTT GAT GCC CCA
Arg Ser Gly Leu Val Asp Ala Ser Gly Val Ala Ser Leu Leu Ala Thr Thr Glu Val Ala Ile Val Asp Ala Pro

+1651 GAA CCA CCA CCA GCT GCT GGC GCT GGT GGT ATG CCA GGT GGT ATG CCA GGA ATG CCA GCT ATG ATG TAA GCACGGC
Glu Pro Pro Ala Ala Ala Gly Ala Gly Gly Met Pro Gly Gly Met Pro Gly Met Pro Gly Met Met >>>

+1727 CTTAATTCAAAATTTATCTTCTTTAAATATGCTTAATAATTTTATTATCTTCTAAAT - 3'

Fig. 3 Nucleotide sequence of the *S. cerevisiae* gene *HSP60*. The predicted amino-acid sequence of the hsp60 protein is indicated. The locations of *BclI*, *PstI* and *Sall* recognition sites are indicated for reference to Fig. 1.

Methods. The primary source of DNA for sequence analysis was the 5.3-kb *EcoRI* fragment shown in Fig. 1. Segments of this fragment were subcloned into pUC118 or pUC119 and sequenced by the chain-termination method²⁴. Subfragments for sequence analysis were generated by exonucleolytic digestion with *Bal31* and recloned into pUC vectors. The sequences of both DNA strands were determined, and all restriction sites used for subcloning were crossed.

Cheng *et al.*⁸ have described a conditional-lethal mutation of yeast, *ts143*, which at non-permissive temperature prevents assembly of multimeric enzymes within mitochondria. To determine whether this assembly defect was due to alteration of hsp60 we tested for allelism between *ts143* and *hsp60::HIS3*. Complementation of the two mutations was assayed directly in diploids as follows. The *ura3⁻/ura3⁻* diploid a/ α W303 ∇ HSP60 was transformed with YEpHSP60; in subsequent manipulations the presence of extrachromosomal *HSP60* is marked by the *URA3* gene of the plasmid. Tetrad dissection gave haploids containing either functional *HSP60* or the null allele *hsp60::HIS3*, along with extrachromosomal YEpHSP60. Each type of haploid was mated to a strain containing *ts143* and diploids were selected by complementation of auxotrophies. As

expected, the *HSP60/ts143* diploid frequently lost YEpHSP60 during mitotic growth, and these uracil-requiring segregants were viable at non-permissive temperature. In contrast, 100 per cent of mitotic segregants from a *hsp60::HIS3/ts143* diploid grown at non-permissive temperature were uracil prototrophs and thus contained extrachromosomal copies of *HSP60*. Therefore, *hsp60::HIS3* and *ts143* do not complement one another and, by definition, are located in the same genetic element. Using a different genetic approach Cheng *et al.*⁸ independently have shown that *ts143* is an allele of *HSP60*.

Hsp60 is the second member of the heat-shock regulon shown to be required for biogenesis of mitochondrial enzymes; hsp70 proteins were recently shown to provide an 'unfolding' function necessary for translocation into both mitochondria and the

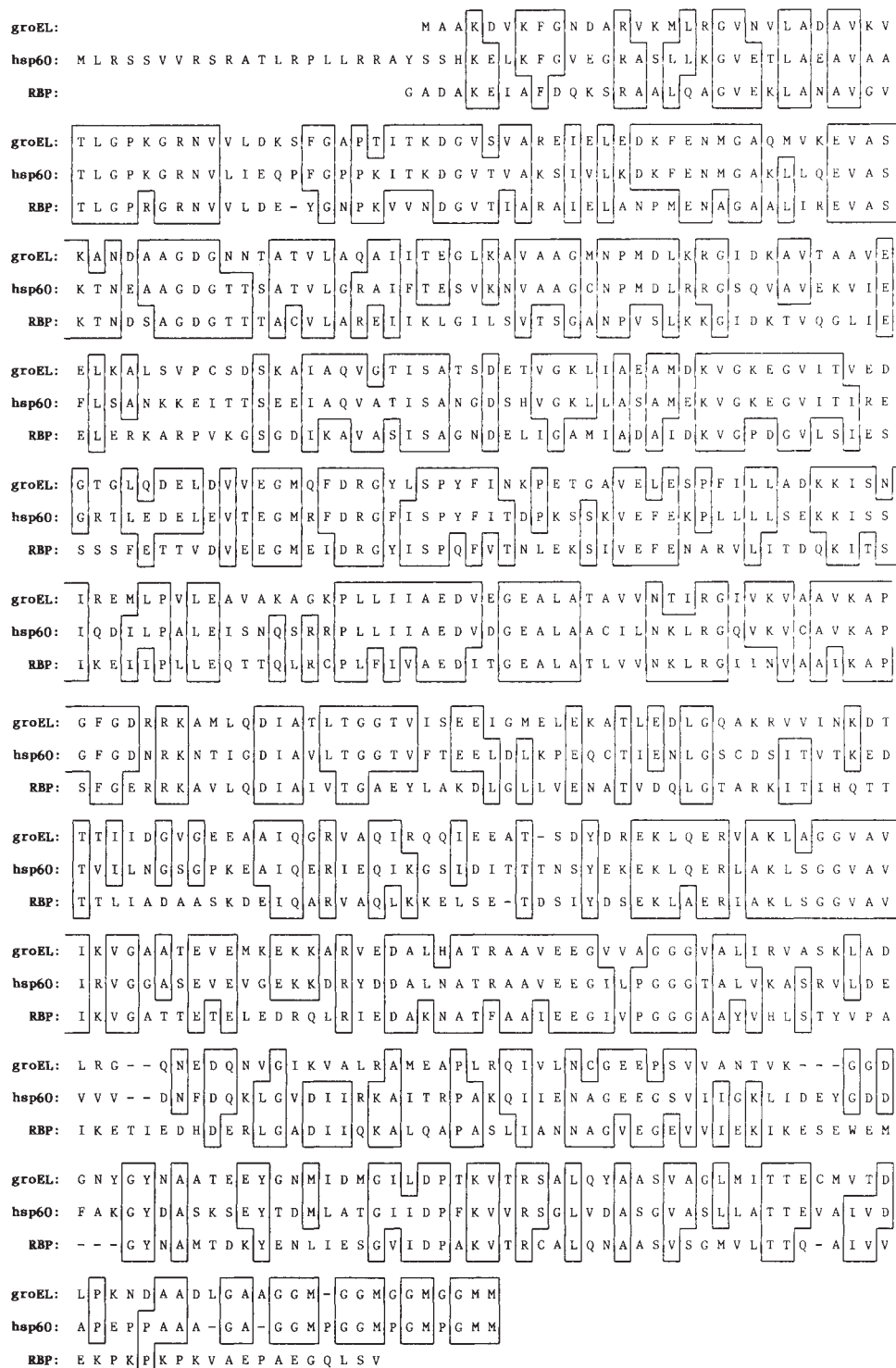


Fig. 4 Primary sequence alignment of yeast mitochondrial hsp60 with *E. coli* groEL and wheat RBP. The predicted amino-acid sequence of the *HSP60* gene product was aligned with the predicted sequences of the groEL and RBP proteins⁷ using the MFALGO program²⁵. Residues shared between hsp60 and either groEL or RBP are boxed.

lumen of the endoplasmic reticulum^{18,19}. Thus, proteins with the ability to influence higher-order structure of various mitochondrial polypeptides are located on both sides of the organelle membrane. The accumulating evidence suggests that such 'unfoldase/refoldase' proteins are widely distributed in prokaryotic and eukaryotic cells.

We thank A. Horwich for supplying the *ts143* strain $\alpha 143$ -3A and for communicating results before publication, and E. Hallberg for technical assistance. This research was supported by the NIH, the Department of Energy and the NSF.

Received 11 November 1988; accepted 16 January 1989.

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Inhibition of thyroid hormone action by a non-hormone binding c-erbA protein generated by alternative mRNA splicing

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Thyroid hormone (T3) binds to a nuclear receptor protein which regulates gene expression by binding to specific DNA sequences near hormone-responsive genes¹. Proteins encoded by two cellular proto-oncogenes, *c-erbA* α and β , bind T3^{2,3} and can act as functional T3 receptors^{4,5}. In rats, alternative splicing of the α -gene transcript generates at least two distinct protein products, termed r-erbA α 1 and r-erbA α 2⁵⁻⁷. Although these proteins bind to the same DNA sequence, r-erbA α 2 does not bind T3. We show here that expression of r-erbA α 2 inhibits the T3-dependent inductive effect of either r-erbA β or r-erbA α 1 on expression of a T3-responsive test gene. Alternative splicing of the *erbA* transcript thus generates products with opposing biological activities, suggesting a novel mechanism for the modulation of hormonal responsiveness.

The structures of the r-erbA β , r-erbA α 1 and r-erbA α 2 proteins studied here are shown schematically in Fig. 1. The human choriocarcinoma cell line JEG-3 was chosen to assess the biological activity of these proteins using cotransfections of appropriate expression vectors with a T3-responsive test plasmid. As is the case with COS^{4,8} and HeLa⁵ cells, the addition of functional T3 receptors to JEG-3 cells by transient transfection results in a gain of hormonal responsiveness.

In the test plasmid pTK14AA, two tandem copies of a modified version of the rat growth hormone (rGH)-T3-response element (TRE)^{4,8} are inserted just upstream of the herpes simplex virus thymidine kinase (TK) promoter in a chloramphenicol acetyltransferase (CAT) vector⁹. As an internal control, all cotransfections also included pTKGH, in which the TK promoter directs expression of human growth hormone

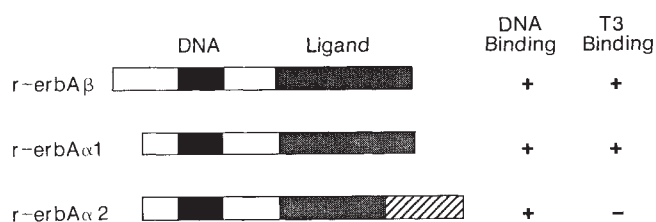


Fig. 1 Schematic representation of r-erbA α 1, r-erbA α 2 and r-erbA β deduced protein sequences. DNA- and hormone-binding domains are assigned on the basis of sequence similarities with steroid receptors, and DNA- and T3-binding capacities are indicated. r-erbA α 1 contains 410 amino acids, r-erbA α 2 contains 492 and r-erbA β contains 461. r-erbA α 1 and r-erbA α 2 are identical in their first 370 amino acids, but then diverge completely. The N-terminal regions of r-erbA α 1 and r-erbA β show no apparent similarity, whereas the putative DNA- and ligand-binding domains show 88% and 82% amino-acid identity, respectively. The β cDNA used here has been described previously⁴. The α 1 cDNA used⁶ is essentially identical to one isolated from rat brain²⁰. The α 2 cDNA used⁶ is essentially identical to cDNAs isolated from rat heart⁵ or brain⁷, that encode proteins that do not bind T3 or its analogues. The *in vitro* translated products of all three rat *erbA* proteins bind specifically to the rGH TRE^{5,6,21} which is also bound by T3 receptors partially purified from rat liver^{22,23}. r-erbA α 2 is very similar to proteins encoded by cDNAs isolated from human testis²⁴ and other tissues²⁵ which, however, are reported to bind T3. This discrepancy in hormone binding may be a consequence of several amino-acid differences in the C-terminal extensions of the rat and human proteins²⁶.

(hGH)¹⁰. Neither CAT nor hGH expression was affected by T3 when JEG-3 cells were cotransfected with just these two plasmids. CAT expression was, however, specifically induced by about 10-fold by T3 if cotransfections also included either of the r-erbA expression vectors pCDM β or pCDM α 1 (Fig. 2).

Cotransfection with the α 2 expression vector pCDM α 2 did not have a strong effect on either of the TK promoter plasmids, with or without T3. The vector pCDM α 2 did, however, have a striking inhibitory effect on T3-induced expression when cotransfected with pCDM β . Cotransfection of 5 μ g pCDM α 2 with 1 μ g pCDM β reduced CAT expression in the presence of T3 to nearly basal levels. α 2 was a somewhat less potent inhibitor of the T3 response conferred by α 1: addition of 5 μ g pCDM α 2 to 1 μ g pCDM α 1 lowered CAT expression in the presence of T3 by about 50%. A T3 dose-response study showed that the inhibitory effect of α 2 was not due to an alteration in the affinity of the active T3 receptors for T3. Similar inhibitory effects of pCDM α 2 were also observed in transfections of CV-1 cells.

pCDM α 2 had no effect on the expression of a test plasmid analogous to pTK14AA but containing a mutant TRE. pCDM α 2 also did not inhibit dexamethasone induction of a mouse mammary tumour virus promoter/CAT test plasmid when cotransfected with a glucocorticoid receptor expression vector. Thus, the inhibitory effect of α 2 requires a functional TRE, and is specific for the T3 receptor.

As measured by T3 binding capacity, levels of receptor expression directed by pCDM β and pCDM α 1 were similar, and were not affected by cotransfection with pCDM α 2. Nuclei of populations of JEG-3 cells transfected with either pCDM or pCDM α 2 bound 9.8 fmol T3 per 100 μ g DNA, confirming the inability of r-erbA α 2 translated *in vitro* to bind T3⁵⁻⁷. In transfections with pCDM α 1, T3 binding increased to 25.1 fmol per 100 μ g DNA, and this level was unaltered by cotransfection with a fivefold excess of pCDM α 2 (24.6 fmol T3 per 100 μ g DNA). Nuclear T3 binding following transfection with pCDM β was 21.3 fmol per 100 μ g DNA, and 21.8 fmol per 100 μ g DNA when cotransfected with a fivefold excess of pCDM α 2. Assuming that <10% of the cells are transfected, these results indicate that T3 receptor levels increase by more than 20-fold in cells transfected with either pCDM β or pCDM α 1.

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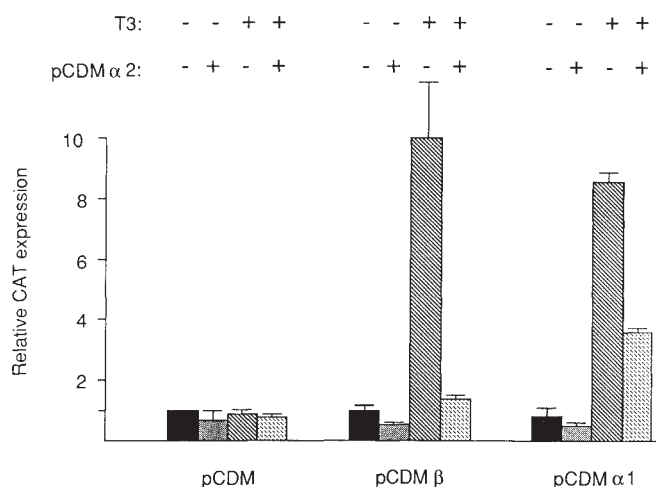


Fig. 2 *r-erbAα2* inhibits T3 induction mediated by *r-erbAβ* or *r-erbAα1*. JEG-3 cells were cotransfected using CaPO_4 as described⁴, with both 4 μg pTK14AA and 2 μg pTKGH, plus either the mammalian expression vector pCDM²⁷ or the indicated combinations of its *r-erbA* expressing derivatives. Northern blots showed that JEG-3 cells express a low level of both $\alpha 1$ - and β -type mRNAs, but no $\alpha 2$ -type mRNA, and confirmed that transfected cells contained levels of CDM-derived $\alpha 2$ -mRNA comparable to the levels of CDM-derived β -mRNA present in cells transfected with pCDM β . As observed with COS^{4,8} and HeLa⁵ cells, the endogenous JEG-3 receptors have no apparent effect on the activity of hormone-responsive test promoters. As results with pituitary cells suggest a correlation between the magnitude of the T3 response and receptor number⁸, the lack of T3 effect may simply be a consequence of the low level of endogenous receptors. Transfections included 1 μg pCDM β or pCDM $\alpha 1$, with or without 5 μg pCDM $\alpha 2$. pCDM was added where necessary to achieve a total of 6 μg of pCDM-based plasmids per transfection. pCDM β is a derivative of a previously described *r-erbAβ* expression vector⁴ containing a deletion removing all upstream AUG codons preceding the open reading frame. pCDM $\alpha 1$ contains a 1,799-base pair (bp) fragment beginning 13 bp upstream of the *r-erbAα1*¹¹ open reading frame, and pCDM $\alpha 2$ contains a 1,947-bp fragment starting at the same position and including the *r-erbAα2*⁶ open reading frame. pTK14AA is a derivative of the CAT expression vector pUTKAT3 (ref. 9), and contains two tandem copies of a modified version of the T3 response element from the rGH gene^{4,8} inserted just upstream of the TK promoter. In the internal control plasmid pTKGH¹⁰, the TK promoter directs human growth hormone (hGH) expression. Relative CAT expression is presented in comparison to that measured in cells cotransfected with pTK14AA, pTKGH and pCDM (no *erbA*-expressing plasmids) and cultured in the absence of T3, arbitrarily defined as 1. To facilitate comparison between transfections, expression was normalized by dividing CAT activities by hGH levels as previously described^{4,8,22,28}.

Methods. Cells were cultured in DMEM with 10% fetal bovine serum. To measure hormone response, individual CaPO_4 precipitates were split into equal parts and used to transfect two plates, which were then cultured in media containing either 10% fetal bovine serum depleted of T3 using charcoal, or the same media supplemented with 5 nM T3. All such media were also supplemented with 100 nM dexamethasone. Growth of JEG-3 cells is not affected by these changes in T3 levels. After two days, CAT activity was measured in cell lysates and hGH levels were measured in media, as previously described⁴. hGH expression was essentially independent of T3 or cotransfected *erbA* plasmids, with typical concentrations ranging from 10–40 ng hGH per 100 μl media, although there was, on average, a slight decrease (approximately 30%) in hGH expression by cells transfected with functional receptors and cultured in the presence of T3. Chloramphenicol acetylation was between 1–5% for cells cultured –T3, and dilutions were made when necessary to maintain acetylation <50% for +T3 cultures. The results are the mean $\pm$ s.e.m. for three separate experiments, each consisting of transfections of two pairs of plates for all combinations of *erbA*-expressing plasmids.

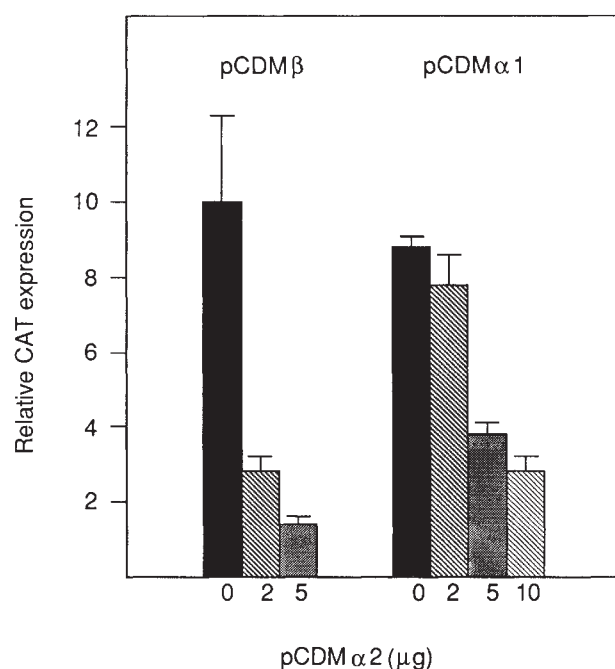


Fig. 3 pCDM $\alpha 2$ inhibits T3-induced CAT expression in a dose-related way. JEG-3 cells were cotransfected with 4 μg pTK14AA, 2 μg pTKGH, 1 μg of either pCDM β or pCDM $\alpha 1$, and the indicated amounts of pCDM $\alpha 2$. Results represent relative CAT expression of cells cultured in the presence of 5 nM T3, in comparison with CAT expression of cells cotransfected with CDM alone in the absence of hormone. The experimental protocol was as described for Fig. 2.

In a dose-response study, a twofold excess of pCDM $\alpha 2$ over pCDM β decreased T3-induced CAT expression by approximately 70%, but higher doses of pCDM $\alpha 2$ were required for similar inhibition of pCDM $\alpha 1$ (Fig. 3). Thus, β is more sensitive than $\alpha 1$ to the inhibitory effect of $\alpha 2$.

To determine whether $\alpha 2$ had an inhibitory effect in T3 responsive cells, the GH4C1 rat pituitary cell line was cotransfected with two different rGH promoter test plasmids and increasing doses of pCDM $\alpha 2$. GH4C1 cells express functional T3 receptors, and contain *r-erbAα1*, *r-erbAβ* and *r-erbAα2* mRNAs^{6,11}. Increasing amounts of pCDM $\alpha 2$ strongly inhibited the relative T3 induction of the test plasmid prGH14A⁸, containing the modified rGH TRE inserted upstream of the rGH promoter. As expected, prGH16A⁸, which contains a deletion specifically removing the TRE, did not respond to T3 in the presence or absence of cotransfected pCDM $\alpha 2$.

We conclude that *r-erbAα2* can inhibit T3 responses generated by *r-erbAβ* and *r-erbAα1*. Several mechanisms could account for this effect: $\alpha 2$ could compete with the functional receptors for binding to the TRE, or could bind to the functional receptors to form inactive heterodimers or hetero-oligomers, or could compete for binding to some accessory factor required for receptor function. Given the range of ligands for the hormone receptor superfamily¹², it is interesting to speculate that the inhibitory effect of $\alpha 2$ could be modulated by binding of an unidentified ligand.

One specific biological role for the variant *r-erbAα2* protein is suggested by the large amount of $\alpha 2$ mRNA in adult rat brain⁶. Although that organ has significant levels of T3 receptors, it does not respond to the hormone as judged by classic criteria^{13–15}. Thus, high levels of $\alpha 2$ may interfere with T3 effects on expression of some genes in the brain.

Inhibition of hormone action by a splicing variant of a receptor is a novel way to modulate response. Opposing effects on gene expression have also been observed for alternatively spliced

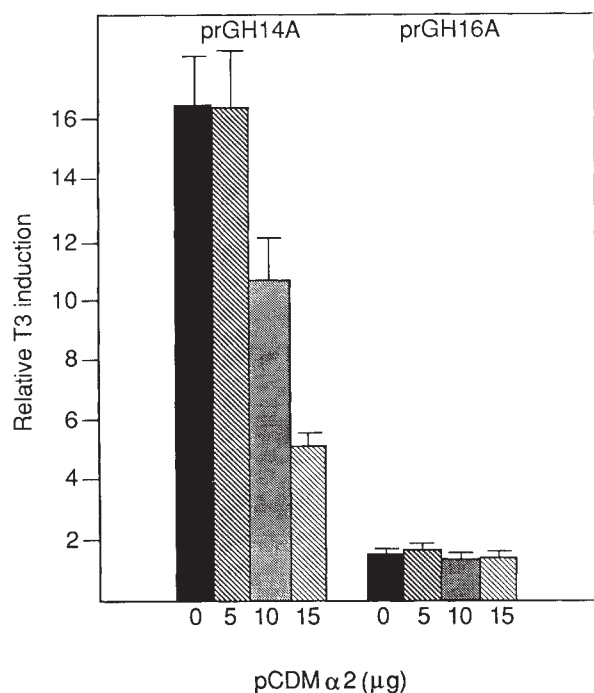


Fig. 4 pCDM α 2 inhibits the activity of the endogenous T3 receptors in rat pituitary cells. GH4C1 cells were transfected using CaPO₄ as described²², with 5 μ g of the CAT expressing test plasmid, 3 μ g of the hGH-expressing internal control plasmid, and the indicated amounts of pCDM α 2. In all transfections pCDM was added as necessary to make a total of 15 μ g of CDM derived plasmid per plate. Results are expressed as relative T3 induction, determined with prGH14A or prGH16A cotransfected with pCDM, in the presence and absence of T3. prGH14A and prGH16A have been described¹² and are, respectively, derivatives of the minimal rGH promoter vector prGH137 (refs 22, 28) containing oligonucleotide inserts of either the modified rGH TRE⁴ or an inactive deletion mutant⁸. Cells were incubated and CAT and hGH were assayed as described in the Fig. 2 legend, except that the medium was Ham's F10.

products of viral genes^{16,17}, and recent results suggest that alternative splicing may generate diversity in the CTF/NF-1 (CCAAT-binding transcription factors/nuclear factor I) class of mammalian transcription factors^{18,19}. Expression of transcription factors with different functions by alternative splicing may be an important general regulatory mechanism.

We thank Robert Warne and John Harney for help with transfections. This work was supported in part by a grant from Hoechst AG to Massachusetts General Hospital. G.A.B. is supported by Physician Scientist Award from the NIH.

Received 7 October 1988; accepted 3 January 1989.

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Prolonged activation of *jun* and collagenase genes by tumour necrosis factor- α

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Tumour necrosis factor- α (TNF- α) is secreted by macrophages in response to inflammation, infection and cancer¹. Sublethal doses of recombinant TNF- α to rats causes cachexia, anaemia and inflammation². TNF- α plays a major part in tissue inflammation³ and remodelling⁴ by stimulating production of collagenase. Cellular responses to TNF- α are initiated by binding to high-affinity cell surface receptors^{5,6}. TNF- α then profoundly affects gene regulation, stimulating the *fos*, *myc*, interleukin-1 and interleukin-6 genes⁷⁻⁹ and inhibiting the type I collagen gene¹⁰. Here we demonstrate that TNF- α also stimulates collagenase gene transcription; this stimulation is mediated by an element of the gene that is responsive to the transcription factor AP-1, the major component of which (*jun*/AP-1) is encoded by the *jun* gene; and that TNF- α stimulates prolonged activation of *jun* gene expression. This prolonged induction of *jun* contrasts with its transient activation by the phorbol ester TPA and provides a physiological example of the ability of *jun*/AP-1 to stimulate its own transcription¹¹. This may be a key mechanism for mediating at least some of the biological effects of TNF- α .

Addition of TNF- α to confluent human fibroblasts causes specific induction of collagenase messenger RNA (Fig. 1a). In these cells TNF- α does not stimulate incorporation of [³H]thymidine¹⁰, which enables us to dissociate stimulation of gene expression from a mitogenic effect. Collagenase mRNA can also be induced by TPA (12-O-tetradecanoylphorbol-13-acetate)¹², but not by other effectors of signal transduction, including Ca²⁺ ionophores, cyclic AMP, prostaglandins or arachidonic acid (data not shown).

Inhibition of protein synthesis (95%) by cycloheximide (0.1 mM) blocks the induction of collagenase mRNA by TNF- α (Fig. 1b, lane 3). By itself cycloheximide leads to low-level induction, probably by stabilizing collagenase mRNA (Fig. 1b, lane 4) and is not toxic to the cells. Thus *de novo* protein synthesis appears to be required for prolonged activation of collagenase gene expression by TNF- α . Transcriptional run-off assays using isolated nuclei indicate that TNF- α increases collagenase gene transcription fourfold, but that it has no effect on activity of the constitutively expressed α -tubulin gene (Fig. 1c). This increased collagenase gene transcription therefore apparently accounts for induction of collagenase mRNA by TNF- α .

Chimaeric plasmids containing segments of the collagenase 5' flanking region ligated to the chloramphenicol transferase (CAT) gene were used to identify the TNF- α -responsive cis-acting element. CAT activity was first assayed in HepG2 cells, which are efficiently transfected¹² and respond to TNF- α ¹³. TNF- α stimulates expression of constructs containing collagenase sequences from -1,200 to +63 or from -517 to +63

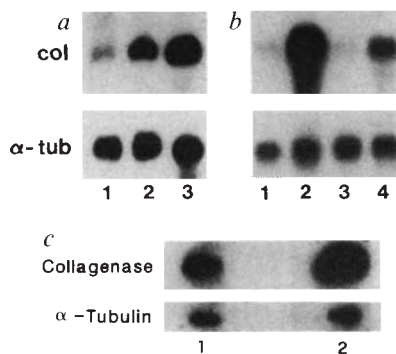


Fig. 1 Analysis of the effects of TNF- α on collagenase gene expression. *a*, Confluent primary human AF2 fibroblast cultures were incubated with human recombinant TNF- α (AmGen) (0.6 nM) for 0, 6 or 24 h (lanes 1, 2 and 3 respectively). Blots of RNA extracted from these cells were hybridized to radiolabelled complementary DNA probes of human fibroblast collagenase¹² (col) or human α -tubulin (α -tub)³³. *b*, Confluent AF2 cultures were incubated for 24 h with no additions (lane 1); TNF- α (0.6 nM) (lane 2); TNF- α (0.6 nM) plus cycloheximide (0.1 mM) (lane 3), and cycloheximide alone (0.1 mM) (lane 4) before extraction of total cellular RNA. *c*, Nuclear run-off transcription analysis of TNF- α -stimulated collagenase gene expression in human fibroblast cultures. AF2 fibroblasts were grown to confluence in roller bottles and incubated either without (1) or with (2) TNF- α (0.6 nM) for 24 h. Nuclei were isolated and *in vitro* run-off transcription performed as previously described^{9,34}. The ³²P radiolabelled nuclear RNA was extracted and hybridized to purified cDNAs that were slot-blotted on to nitrocellulose filters.

Methods. Human fetal AF2 fibroblasts were cultured under an atmosphere of 5% CO₂/95% air in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum. Cells were plated at a density of 9×10^5 /P-100 dish and subjected to the various treatments after 6 days. Total cellular RNA was extracted¹⁰. Purified fragments of collagenase¹² and tubulin³³ cDNAs were radiolabelled with α -³²P-dCTP using random-primer synthesis to a specific activity of $\sim 1 \times 10^9$ c.p.m. per μ g DNA. Northern blots and slot blots on nylon filters have been described previously¹⁰. Slot-blot autoradiographs were quantitized by a scanning laser densitometer interfaced with an integrator.

(Fig. 2a); these sequences contain the TPA-responsive element (TRE) of the collagenase gene, consisting of eight base pairs (−73 to −65) constituting a binding site for transcription factor AP-1 (refs 12 and 14). TNF- α enhances the activity of constructs containing one or five copies of the TRE upstream of the herpes simplex virus-tk promoter (1 $\times$ TRE-CAT, 5 $\times$ TRE-CAT) by threefold and seventeenfold respectively. Neither TNF- α nor TPA stimulates the activity of tk-CAT or of Δ -72 TRE-CAT, which contains a point mutation that interferes with the binding of AP-1 to the TRE¹⁴. Stimulation of 5 $\times$ TRE-CAT activity by TNF- α results from increased production of correctly initiated CAT mRNA (Fig. 2c). We confirmed these results in primary human fibroblasts, which are physiologically more relevant, but also more refractory to transfection¹⁵. TNF- α and TPA also stimulate CAT activity from 5 $\times$ TRE-CAT, but not from tk-CAT (Fig. 2b), as expected. Therefore an intact AP-1 recognition sequence is necessary and sufficient for induction of CAT activity by both TNF- α and TPA in either HepG2 cells or primary human fibroblasts.

As induction of collagenase by TNF- α is dependent on *de novo* protein synthesis, we investigated whether this is associated with activation of the *jun* gene that encodes the main component of purified AP-1 known as jun/AP-1 (refs 16–18). Incubating fibroblasts with TNF- α results in prolonged induction of *jun* mRNA lasting at least six hours (Fig. 3a). However, the effect of TNF- α on *jun* transcription is reversible, and by 24 hours *jun* mRNA returns to its basal level (data not shown). In contrast, TPA treatment results in a more transient increase in the *jun* mRNA level, with maximum stimulation after 1 h

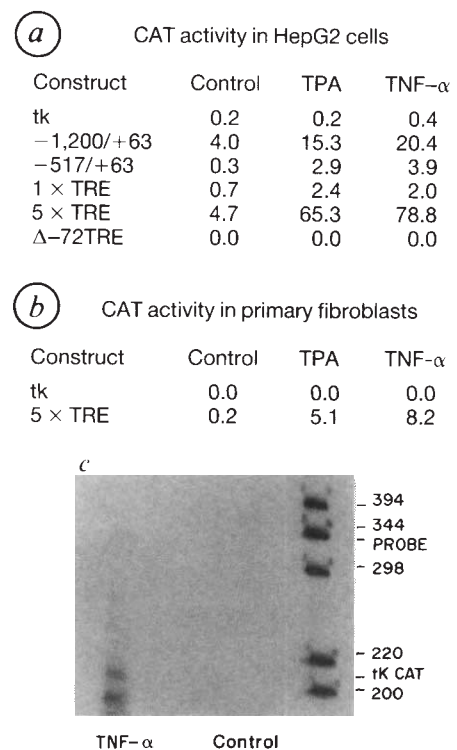


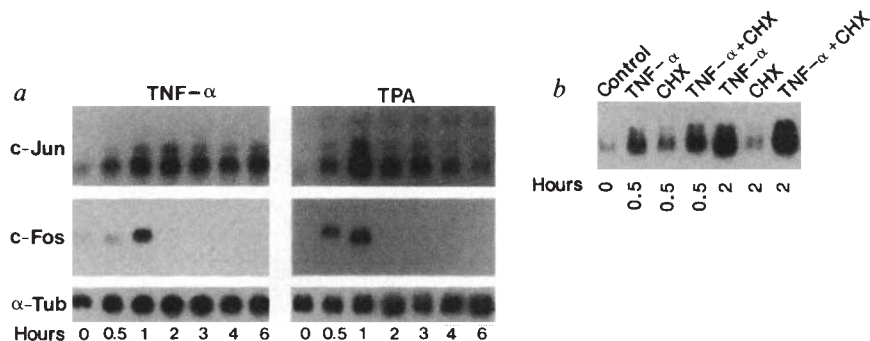
Fig. 2 Stimulation of genes containing collagenase gene segments by TNF- α . The effect of TPA and TNF- α on the chloramphenicol acetyl transferase activity of transiently transfected HepG2 cells (*a*) or AF2 primary human fibroblasts (*b*). Supercoiled plasmids (tk-CAT, −1,200/+63 collagenase-CAT, −517/+63 collagenase-CAT, 5 $\times$ TRE-CAT, and Δ -72 TRE-CAT) were introduced into HepG2 cells¹² and into primary human fibroblasts using the Chen-Okayama method¹⁵. The cells were then incubated for 13 h either with no additions, or with TPA (20 ng ml^{−1}) or TNF- α (0.6 nM), and assayed for CAT activity^{12,35}. CAT activity is expressed as per cent acetylation of chloramphenicol and represents the mean of triplicate experiments. *c*, RNase protection analysis of collagenase-CAT mRNA. HepG2 cells were transfected with 5 $\times$ TRE-CAT and incubated with no additions (control) or with TNF- α (0.6 nM). Isolated total RNA (20 μ g) was hybridized with 5×10^5 c.p.m. radiolabelled SP6 RNA probe³⁶ and digested with RNases¹⁴. Size markers (in base pairs) are shown to the right.

(Fig. 3a). Induction of *jun* mRNA by TNF- α is not blocked by cycloheximide (Fig. 3b), implying it does not require *de novo* protein synthesis.

As the fos protein interacts with jun/AP-1 to form a more effective transcriptional activator operating through the AP-1 binding site^{18,19}, we determined the effect of TNF- α on *fos* mRNA levels in these cultured fibroblasts. TNF- α or TPA elicit a rapid and transient induction of *fos* mRNA, with virtually no *fos* mRNA being detectable by 2 h (Fig. 3a). The increase in *jun* and *fos* mRNA is paralleled by an increase in immunoprecipitable jun/AP-1 and fos protein synthesis (data not shown). Therefore, TNF- α stimulates the expression of both *fos* and *jun* genes, whose products interact to stimulate transcription of AP-1-responsive genes.

Most of the known effects of phorbol esters, including stimulation of gene transcription, are mediated by activation of protein kinase C (PKC)^{20–22}. Activated PKC associates in a Ca²⁺-independent manner with the plasma membrane to phosphorylate membrane proteins²¹. Two independent PKC assays demonstrated that plasma membrane-PKC activity is stimulated by incubating the fibroblasts with either TPA or TNF- α (Fig. 4). In addition, the PKC inhibitor H7 (ref. 23) inhibits induction of collagenase mRNA by either TPA or TNF- α . But prolonged coincubation with TNF- α and H7 is associated with some cel-

Fig. 3 Northern blot analysis of the effects of TNF- α and TPA on *jun* and *fos* expression. *a*, Confluent AF2 cultures were incubated with TNF- α (0.6 nM) or TPA (20 ng ml⁻¹) for 0, 0.5, 1, 2, 3, 4 or 6 h after which total cellular RNA was extracted. Blots of RNA from these cells were hybridized to radiolabelled *jun*¹⁴ or α -tubulin³³ cDNA probes. *b*, Confluent AF2 cultures were incubated either with no additions (control) with TNF- α (0.6 nM), with cycloheximide (CHX; 0.1 mM) or with TNF- α (0.6 nM) plus cycloheximide (0.1 mM) for 0, 0.5 or 2 h. RNA blots were hybridized to a *jun* cDNA probe.



lular toxicity (data not shown). We conclude that like TPA, TNF- α activates PKC which in turn may result in increased AP-1 activity and induction of AP-1-responsive genes.

Epidermal growth factor²⁴ and serum²⁵ have been shown to cause transient induction of *jun* mRNA in immortal cells undergoing mitosis, which differs from the prolonged activation of gene expression in quiescent primary human fibroblast cultures shown here. Such cells are likely to reflect one of the natural targets for TNF- α action. The stimulation of *jun* and collagenase gene expression by TNF- α is prolonged in comparison to the more transient effect of TPA^{11,26}. On the other hand, the kinetics of *fos* activation by both agents are identical. It is possible that TNF- α does not lead to subsequent down-regulation of PKC as rapidly as TPA (ref. 22). The *jun* gene product stimulates the transcription of its own gene through an AP-1 binding site in the *jun* promoter¹¹; under certain circumstances, this positive

autoregulatory mechanism could lead to subsequent prolonged stimulation of *jun* expression. It has been proposed that the autostimulatory activity of the *jun* gene plays a key part in converting transient signals generated by occupied cell surface receptors to long-lasting effects on cellular gene expression^{22,27,28}, a proposition which is supported by our results.

An apparent paradox is that a 'tumour necrosis factor' stimulates the expression of proto-oncogenes. In reality, TNF- α has a variety of effects on cell proliferation, including to act as a growth factor for normal fibroblasts²⁹ in both normal³⁰ and transformed³¹ B cells. Bacterial extracts, such as *Bacillus Calmette-Guerin* cell-wall preparations, that stimulate TNF- α production can actually enhance tumour growth³². Perhaps these proliferative responses result from the stimulation of proto-oncogene expression by TNF- α .

This work was supported by the US Public Health Service (D.A.B., M.C. and M.K.) and the Veterans Administration (D.A.B. and M.C.) and the Deutscher Akademischer Austauschdienst (P.A.). We thank M. Filip for technical assistance, B. Brelin for graphics and A. Arata for typing the manuscript.

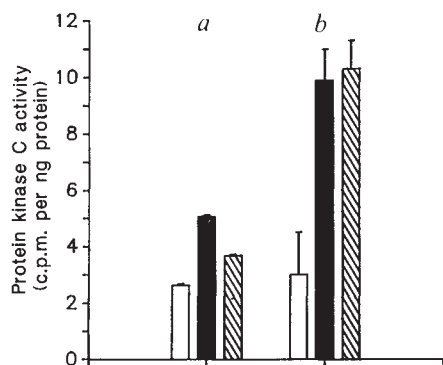


Fig. 4 Stimulation of protein kinase C activity in human fibroblast plasma membranes. Confluent AF2 cultures were incubated with no additions (control; open bars), with TPA (filled bars) or TNF- α (cross-hatched bars). Two different assays demonstrated specific activation of PKC based on its requirement for phospholipids (*a*) or its inhibition by H7 (*b*).

Methods. Confluent AF2 cells (5.6×10^6) were incubated in DMEM containing 10% fetal calf serum for 30 min at 37 °C with no additions (control) or with either TPA (20 ng ml⁻¹) or TNF- α (1.2 nM). Cells were lysed and the plasma membrane fraction purified by centrifugation at 100,000g. PKC was extracted from plasma membrane in 20 mM Tris, pH 7.5, 0.33 M sucrose, 0.5 mM EGTA, 2 mM EDTA, 2 mM PMSF and 1% NP40 and its activity determined using histone III-S as a substrate for phosphorylation^{20,22}. *a*, Specific PKC activity represents ³²P c.p.m. incorporated into histone in the presence of phospholipids (0.9 mM dioleoin and 0.5 mg ml⁻¹ phosphatidyl serine), minus that incorporated in their absence. *b*, Specific PKC activity represents ³²P c.p.m. incorporated into histone in the presence of phospholipids alone, minus that incorporated after the addition of the PKC inhibitor H7. Values represent mean $\pm$ s.e.; $P < 0.02$ for TPA and TNF- α compared with control values.

Received 30 August; accepted 16 December 1988.

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Involvement of the 'leucine zipper' region in the oligomerization and transforming activity of human c-myc protein

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c-Myc plays a part in the regulation of important cellular processes such as growth, differentiation and neoplastic transformation¹⁻³. Although c-myc gene structure and expression are well characterized¹⁻³, the function and biochemical properties of the protein are less well understood⁴⁻⁶. Human c-myc is a 439-amino acid phosphoprotein which binds DNA *in vitro* and belongs to a discrete subset of nuclear proteins¹⁻³. Using the human c-myc mutants generated by linker-insertion and deletion mutagenesis, we have defined regions of the protein that are important for its transforming activities⁷ and its nuclear localization⁸. Here, we show that human c-myc exists as an oligomer *in vitro* and use mutant proteins to localize the oligomerization domain to a carboxyl-terminal peptide containing the 'leucine zipper' motif. The 'leucine zipper' describes a structure found in a number of DNA-binding proteins that contains leucines occurring at intervals of every seventh amino acid in a region predicted to be α -helical⁹. The 'leucine zipper' might mediate dimerization by intermolecular interdigitation of the leucine side-chains. We show that a c-myc mutant, which is inactive but can oligomerize, dominantly inhibits the cotransforming activity with wild-type c-myc of rat embryo cells, whereas inactive mutants which cannot oligomerize properly because of deletions in the oligomerization domain are recessive.

To study the characteristics of c-myc, we used bacterially synthesized wild-type and mutant human c-myc in the pOTSmc plasmid and its derivatives, produced by subcloning c-myc fragments containing mutations into the pOTS vector¹⁰ (a gift from R. Watt and A. Shatzman of Smith, Kline and French). Deletion mutants are designated 'D' followed by the positions of the deleted codons. Highly purified wild-type c-myc behaves in gel filtration chromatography as a protein of relative molecular mass (M_r) 200,000 (200K) (Stokes' radius, 4.7 nm) (Fig. 1a). The

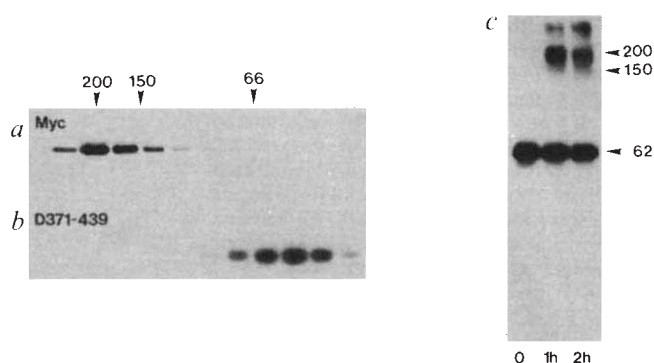


Fig. 1 Immunoblots of fractions from gel filtration chromatography of wild-type and mutant c-myc recombinant proteins. M_r values (K) for marker proteins are shown above the panels a and b: aldolase (200K), yeast alcohol dehydrogenase (150K) and bovine serum albumin (66K). a, The purified wild-type c-myc 62K polypeptide elutes as an oligomer of M_r 200K, whereas a mutant 52K polypeptide that is deleted from amino acids 371-439 is monomeric (b). c, Immunoblot of glutaraldehyde-cross-linked products of purified c-myc protein. M_r s of polypeptides are shown on the right (K) and incubation times (hours) at the bottom.

Methods. Mutant c-myc complementary DNAs in the pOTS vector were generated by subcloning *Bst*EII-*Hind*III fragments of mutant c-myc cDNAs⁷ into a modified pOTSmc vector. Recombinant wild-type and mutant c-myc protein were purified as described¹⁶. Protein samples (150 μ l) were eluted with TBS at 6.0 ml h^{-1} at 4 °C in a 0.9 $\times$ 30 cm Sephacryl S300 (Pharmacia) column. Polypeptides in column fractions (0.5 ml) were immunoblotted after SDS-PAGE^{18,19} and visualized with rabbit anti-recombinant-c-myc antibody and ¹²⁵I-labelled protein A. M_r s of polypeptides were estimated from prestained protein markers (Biorad). c, A 0.75 μ M solution of purified c-myc protein was incubated with 0.005% glutaraldehyde in 75 mM sodium phosphate buffer, pH 7.0, at 20 °C. Samples were removed at different times, electrophoresed on SDS-polyacrylamide gels and immunoblotted.

sedimentation coefficient of c-myc is estimated to be 10.5 S by velocity sedimentation in 10-30% glycerol gradients (data not shown). The derived native relative molecular mass of c-myc is 213K, which is consistent with a tetramer¹¹ (Table 1). The oligomerization of c-myc was also studied by chemical cross-linking (Fig. 1c). The dominant cross-linked species has an estimated M_r of 200K by SDS-PAGE, which is also consistent

Table 1 Estimated M_r and subunit structure of wild-type and mutant c-myc proteins and of staphylococcal protein A/c-myc fusions

Protein*	s (S)†	R_s (nm)‡	Native M_r §	Subunit M_r §	Structure
c-myc	10.5	4.7	213,000	62,000	tetramer
D106-143	8.5	4.5	169,000	54,000	tetramer
D371-412	6.2	4.0	107,000	55,000	dimer
D414-433	4.0	3.2	55,000	54,000	monomer
PA[372-439] myc	4.3	4.6	85,000	35,000	dimer
PA[262-326] myc	2.2	3.5	33,000	35,000	monomer
Protein A	2.1	5.0	45,000	45,000	monomer

* c-myc mutants are designated 'D' followed by position numbers of the deleted amino acids. Using the one-letter code, the carboxyl-terminal c-myc 72 amino acids are as follows, with the leucines in heptad repeat underlined: 369-ELKRSFFALRDQIPELENNEKAPKVVLKKATAYILSVQAEQKLISEEDLLRKRRQLKAKLEQLRNSCA. The 'leucine zipper' region includes leucines at residues 413, 420, 427 and 434. Protein A/c-myc fusion proteins are designated by the positions of the included c-myc amino acids in brackets.

† Sedimentation coefficients are estimated from peak fractions of 10-30% glycerol gradients in TBS (10 mM Tris-HCl, pH7, 150 mM NaCl).

‡ Stokes' radii.

§ Native M_r s are estimated from the sedimentation coefficients and Stokes' radii using standard hydrodynamic equations¹¹. Subunit M_r s are estimated from SDS-polyacrylamide gels (note that c-myc migrates slowly and has a predicted M_r of 49K).

|| Values are from ref. 13.

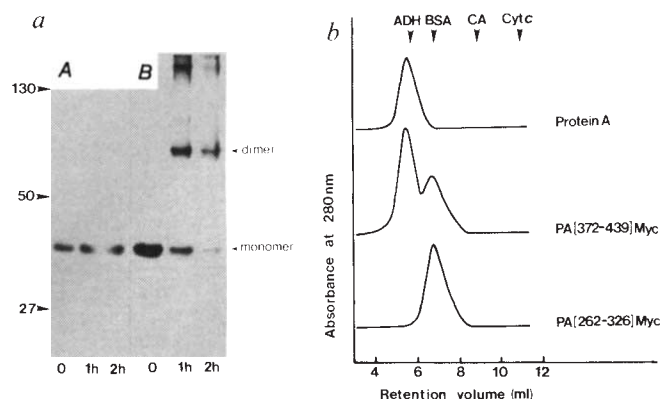


Fig. 2 *a*, Immunoblots of glutaraldehyde-cross-linked products of staphylococcal protein A/c-myc fusion proteins. The included c-myc amino acids are A, 262–326, B, 372–439. Incubation times are shown below the panels and protein size markers to the left (K). *b*, FPLC gel filtration chromatography of purified protein A/myc fusion proteins using a Superose 12 column (column volume, 25 ml). Positions of marker proteins eluting in order of decreasing Stokes' radius are given at the top: yeast alcohol dehydrogenase (ADH; R_s 4.5 nm); bovine serum albumin (BSA; R_s 3.5 nm); bovine erythrocyte carbonic anhydrase (CA; R_s 2.4 nm) and cytochrome *c* (Cyt; R_s 1.7 nm).

Methods *a*, The fusion genes were constructed using the pRIT2T protein A bacterial expression vector and the fusion proteins were purified as described^{12,20}. Purified proteins (1 μ M) were treated with glutaraldehyde as in Fig. 1c. *b*, Protein samples of 200 μ l (1–2 mg ml⁻¹) were injected and eluted with TBS at a flow rate of 0.5 ml min⁻¹ (20 °C). Fraction volume was 0.5 ml.

with a tetrameric form; minor species migrating with M_r 150K and M_r > 250K are also present. The unreacted subunit runs at 62K. Thus by three independent methods, we have found that human c-myc exists as an oligomer *in vitro*.

To test whether loss of the c-myc carboxy-terminal region containing the 'leucine zipper' motif (see Table 1 footnote) prevents oligomerization, we determined the native M_r s of c-myc mutants that are deleted in this region. A mutant protein (D371–439) that is deleted at the C-terminal by 69 residues behaves as a monomeric protein of M_r 50K by gel filtration (Fig. 1b). To analyse this region further, we examined the gel filtration characteristics of mutant myc containing smaller deletions. D371–412 yields a 55K protein which has an M_r of slightly less than 150K in the native form, indicating the presence of dimers (Table 1). D414–433 totally disrupts the 'leucine zipper' motif and behaves as a monomeric protein with native M_r = 55K (Table 1). As a control, deletion of residues 106–143 (another region required for myc transforming activity⁷) does not affect oligomerization (Table 1). These gel filtration results were confirmed by velocity sedimentation. The intact D371–412 protein sediments at 6.2 S, indicative of a dimer form (Table 1), whereas D414–433 sediments at 4.0 S, indicating that monomers are present (Table 1). Thus, both our gel filtration and velocity sedimentation results indicate that disruption of the 'leucine zipper' motif generates monomeric proteins, whereas disruption of the amino-acid region 371–412, which does not involve the 'leucine zipper', still permits dimerization of myc.

If C-terminal myc amino acids are involved in oligomerization, this region might behave as a discrete functional domain. Therefore we constructed genes that fuse the 28K N-terminal region of staphylococcal protein A to regions of human myc to determine whether the C-terminal c-myc region could promote oligomerization of the hybrid protein¹². Proteins with c-myc amino acids 262–326 or 372–439 fused to protein A were purified to >95% homogeneity. These proteins are designated PA fol-

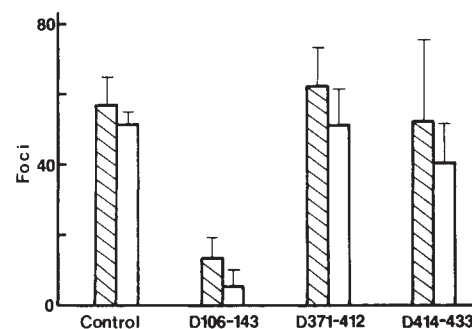


Fig. 3 Effects of defective c-myc mutants on the wild-type c-myc cotransformation activity of rat embryo cells. Rat embryo cells were transfected with EJras (10 μ g) and wild-type c-myc (10 μ g, cross-hatched; 3 μ g, open) plasmids in the absence (control) or presence of various mutant c-myc (10 μ g) plasmids⁷. The mean numbers of foci per 10⁶ transfected rat embryo cells from three separate experiments are given as vertical bars with vertical lines at the top of the bars depicting standard deviations. Mutant c-myc plasmids alone do not cotransform rat embryo cells with EJras (ref. 7).

lowed by the included c-myc amino acids in brackets. Cross-linking of these fusion proteins using glutaraldehyde indicates that c-myc amino acids 262–326 do not mediate dimerization of the fusion protein (Fig. 2a, panel A). In contrast, the fusion protein containing the c-myc 'leucine zipper' yields dimers and oligomers after cross-linking (Fig. 2a, panel B). We further showed that this region of c-myc promotes oligomerization by subjecting the purified proteins to fast protein liquid chromatography (FPLC) (Fig. 2b). Purified protein A (45K) is an asymmetric monomeric molecule with a Stokes' radius of 5.0 nm, in agreement with our observation¹³ (Fig. 2b). The fusion protein containing the 28K N-terminal segment of protein A and the 'leucine zipper' (PA[372–439]myc) displays two peaks which are consistent with the presence of both dimers and monomers (Fig. 2b). The fusion protein containing c-myc amino acids 262–326 exists exclusively as a monomer and elutes at the same position as the smaller monomeric peak of PA[372–439]myc. To confirm the subunit composition of these fusion proteins, native M_r s were estimated from Stokes' radii and sedimentation coefficients (Table 1). These results indicate that the 68 amino acids at the C-terminal of myc can mediate dimerization.

If functional c-myc is multimeric *in vivo*, then an inactive c-myc derivative or *trans*-dominant negative mutant¹⁴ capable of interacting with wild-type polypeptide chains will be inhibitory if it causes the formation of non-functional multimers. We assessed the effects of various inactive c-myc mutants on the ability of wild-type c-myc to cotransform normal rat embryo cells with EJ-ras (ref. 7). Inactive mutants that are oligomerization-defective, namely D371–412 and D414–433, are recessive. But the D106–143 mutant, which lacks transforming activity but preserves the oligomerization domain, can dominantly inhibit the transforming activity of wild-type c-myc (Fig. 3). These observations support the *in vivo* significance of c-myc oligomerization and are consistent with a previous report that v-myc is oligomeric in cellular extracts¹⁵. Although our *in vitro* data indicate the possible existence of c-myc homo-oligomers *in vivo*, the *in vivo* c-myc oligomers could be not dimers or tetramers, but complexed with other proteins like c-jun/c-fos^{16,17} for example.

In summary, we have demonstrated that c-myc synthesized in bacteria exists in oligomeric form and that oligomerization depends on the integrity of a C-terminal region containing the 'leucine zipper' motif⁹. When this region is fused to monomeric

protein A, the hybrid protein dimerizes. Another c-myc region containing amino acids 370–384 may play a part in c-myc oligomerization, because its deletion prevents tetramerization but not dimerization. As we have not performed a point mutation analysis on the 'leucine zipper', our results imply, but do not stringently prove, that the leucine repeat is the critical feature in oligomerization. The integrity of the 'leucine zipper' seems to be essential *in vivo*, as it is required for transforming activity⁷ and as a defective c-myc mutant with an intact oligomerization domain behaves as a dominant negative mutant. Such a negative mutant provides a new tool to impede the activity of wild-type c-myc.

This study was supported by Biomedical Research (C.V.D.), the American Cancer Society Institutional Research (C.V.D.) and by the NIH (W.M.F.L.). W.M.F.L. holds an American Cancer Society junior faculty research award. C.V.D. is a Hubert and Anne E. Rogers Scholar and W. M. Keck Foundation clinician scientist. C.V.D. thanks J. L. Spivak and J. D. Stobo for their support, W. Landschulz and S. L. McKnight for discussion and P. Agre for use of the FPLC system.

Received 14 November; accepted 21 December 1988.

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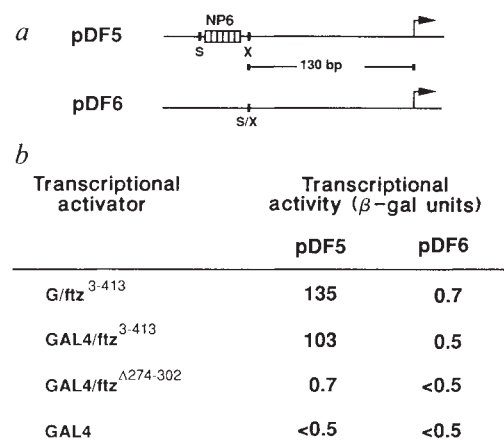


Fig. 2 Consensus binding sites for ftz in *Drosophila* act as a ftz-dependent UAS element in yeast cells. Transcription of β -galactosidase reporter constructs containing either six tandem repeats (NP6) of a consensus ftz-binding site⁸ in *Drosophila* (pDF5) or lacking any UAS element (pDF6) was used to assess transcriptional activation by G/ftz³⁻⁴¹³, GAL4/ftz³⁻⁴¹³, the homoeobox deletion mutant GAL4/ftz ^{Δ 274-302}, or GAL4 itself. **a**, Reporter constructs. **b**, Transcriptional activation.

Methods. The β -galactosidase reporter construct pDF5 containing six tandem copies of the consensus ftz-binding site TCAAT-TAAATGA⁸ is a derivative of the 2 μ URA3 yeast plasmid pLR1 Δ 20 (ref. 29), in which the UAS_G DNA between the unique Sma1 (S) and Xho1(X) sites was replaced by a Sma1–Sal1 fragment from an M13mp18 clone containing the consensus ftz-binding sites. The UAS_G DNA between the Sma1 and Xho1 sites was deleted in construction of pDF6. DNA for these reporter constructs and the indicated GAL4 derivatives was used to transform the yeast strain YM709 (Δ gal80 Δ gal4 *ade2-101 lys2-801 his3-200 met trp tyr can1*) and β -galactosidase assays of yeast cell extracts were performed after growth on minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with the appropriate amino acids. The G/ftz³⁻⁴¹³ construct which expresses a chimaeric GAL4/ftz polypeptide containing amino acids 1–10 of GAL4 and amino acids 3–413 of ftz was made by introducing a 10-base pair EcoR1 linker at the bluntend Sph1 site in GAL4 DNA in the plasmid pMA235 (ref. 14) and then cloning ftz cDNA with EcoR1 linkers between the two EcoR1 sites of this pMA235 derivative. The non-ftz amino acids encoded at the GAL4/ftz junction of this construct are: MKLLSSIEQAEEFGDP-T(3)-ftz.

The *Drosophila* fushi tarazu polypeptide is a DNA-binding transcriptional activator in yeast cells

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Many of the regulatory genes controlling the developmental pattern of segmentation during embryonic development in *Drosophila melanogaster*^{1–3} encode nuclear proteins containing either homoeobox or 'zinc-finger' domains with putative^{4–6} or demonstrated^{7,8} sequence-specific DNA-binding properties. One of these *Drosophila* homoeobox-containing proteins is encoded by the *fushi tarazu* (*ftz*) gene. The expression of *ftz* is spatially restricted during embryogenesis^{9–11} and the ftz polypeptide has an important role in different stages of development^{12,13}. To determine whether the ftz polypeptide is a sequence-specific DNA-binding activator of transcription, we expressed portions of ftz as fusions with the yeast transcription factor GAL4 in yeast cells. Chimaeric GAL4/ftz proteins, like GAL4 itself, activated the transcription of a GAL4-dependent reporter gene. With reporter constructs containing *Drosophila*-derived chromosomal DNA sequences as transcriptional elements, the ftz polypeptide acted as a sequence-

specific DNA-binding transcriptional activator. In *Drosophila*, the ftz product may therefore be a positive regulator of transcription.

To assess the transcriptional activity of ftz in yeast cells, we tested whether there were any domains within the ftz polypeptide that could functionally replace the acidic transcriptional activation domains¹⁴ of GAL4. To do this, we fused the coding sequences of the DNA-binding domain of GAL4 (amino acids 1–147) to cDNA sequences encoding all but the N-terminal methionine and alanine residues of the ftz polypeptide. The resulting GAL4/ftz³⁻⁴¹³ chimaera, like similar fos and myc chimaeras¹⁵, was almost as effective as GAL4 itself in activating transcription in yeast cells of a reporter construct containing GAL4-dependent upstream activating sequence (UAS_G) fused to β -galactosidase coding sequences (Fig. 1a). To map independent DNA-binding and transcriptional activation domains in the ftz polypeptide, we assessed the ability of GAL4/ftz constructs containing deletions in the ftz coding sequence to activate transcription of the same reporter gene (Fig. 1a). The transcriptional activation function was reduced in cells expressing deletion clones lacking the N-terminal 222 amino acids (GAL4/ftz²²³⁻⁴¹³) or C-terminal 141 amino acids (GAL4/ftz³⁻²⁷²) of the ftz polypeptide and was eliminated by more extensive deletions removing the N-terminal 272 amino acids (GAL4/ftz²⁷³⁻⁴¹³) or the C-terminal 192 amino acids (GAL4/ftz³⁻²²¹). The inactive derivatives were not unstable poly-

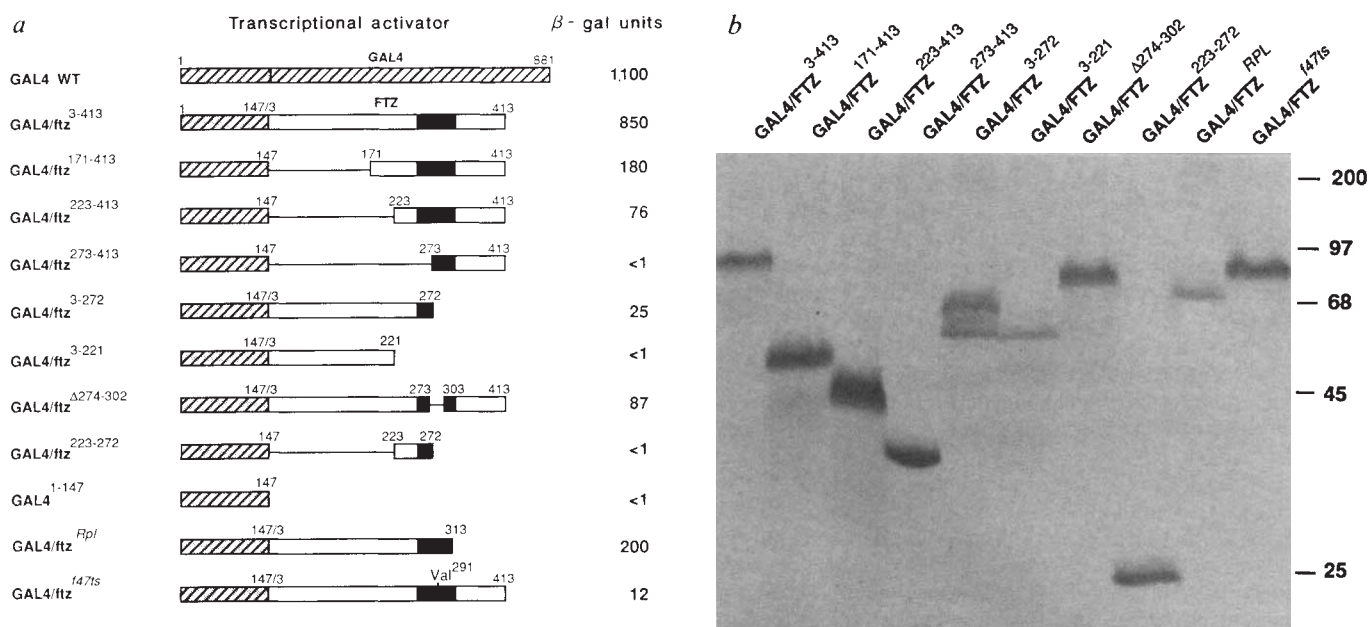


Fig. 1 *a*, UAS_G-dependent transcriptional activation by GAL4/ftz chimaeras. The ability of various GAL4/ftz chimaeric polypeptides expressed in yeast cells to activate transcription was assessed by quantitating β-galactosidase activity present in extracts of yeast cells containing a GAL4-dependent (UAS_G) β-galactosidase reporter gene. The activation of transcription by wild-type GAL4 (GAL4 WT) has been compared with that by GAL4/ftz chimaeras containing amino acids 1–147 of GAL4 and the indicated amino acids of the ftz polypeptide. The filled box denotes the homoeodomain, the hatched boxes are GAL4 sequences. *b*, Immunodetection of GAL4/ftz polypeptides present in yeast cell extracts. Relative molecular masses are shown on the right, in thousands.

Methods. Wild-type GAL4 DNA was introduced into yeast cells on the yeast plasmid pMA210 (ref. 14). The GAL4/ftz chimaeras were constructed by cloning ftz cDNA fragments originating from the plasmid pGEMf1 (ref. 11) into the unique EcoRI site encoding amino acid 148 of GAL4 in the pMA210 derivative pMA235 (ref. 14). GAL4 and the GAL4/ftz chimaeras were therefore expressed from the ADHI promoter on a HIS3 2μ yeast vector as described¹⁴. The correct reading-frame fusions were achieved using an appropriately sized EcoRI linker and/or by first cloning the ftz DNA fragments into the polylinker of pEMBL or Bluescript vectors. The amino acids (one-letter code) at the GAL4/ftz junctions were: for GAL4/ftz³⁻⁴¹³, GAL4/ftz³⁻²⁷², and GAL4/ftz³⁻²²¹, GAL4-S(147)-PEFIKEEKLTMTRDP-T(3)-ftz; for GAL4/ftz¹⁷¹⁻⁴¹³, GAL4-S(147)-PEFELGTRGSSR-V(171)-ftz; for GAL4/ftz²²³⁻⁴¹³ and GAL4/ftz²²³⁻²⁷², GAL4-S(147)-PEFELGTP-V(223)-ftz; for GAL4/ftz²⁷³⁻⁴¹³, GAL4-S(147)-PEFELGTRGSSR-E(273)-ftz; for GAL4/ftz^{Rpl} and GAL4/ftz^{147ts}, GAL4-S(147)-PEFGDP-T(3)-ftz. Amino acids in the C-terminal extensions before the first in-frame stop codon were: for GAL4/ftz³⁻²⁷², L(272)-DREGVPLQ; GAL4/ftz³⁻²²¹, S(221)-GL; and for GAL4¹⁻¹⁴⁷, S(147)-PEF. The GAL4/ftz^{Δ274-302} construct was made by deletion of ftz cDNA between its XhoI and BglII sites. DNA of the GAL4 and GAL4/ftz constructs was used to transform the yeast strain YM709::RY171 (Δgal80 Δgal4 ade2-101 lys2-801 his3-200 met trp tyr can1) which contains a single copy of the GAL1/lacZ reporter RY171 (ref. 26) integrated at the URA3 locus. The ability of the chimaeric constructs to stimulate expression of the reporter gene was quantitated by assessing β-galactosidase activity in the yeast cell extracts²⁷ after growth at 30 °C in minimal medium (0.67% yeast nitrogen base without amino acids, 2% raffinose) supplemented with the appropriate amino acids. The GAL4/ftz^{147ts} strain was grown at 19 °C. For immunodetection of GAL4/ftz polypeptides, protein extracts were prepared as described²⁸ from 5 ml yeast cells grown to mid log-phase. Equivalent aliquots of each protein preparation were fractionated on an 11% polyacrylamide-SDS gel, and electrophoretically transferred on to nitrocellulose. The GAL4/ftz polypeptides were detected with a polyclonal anti-ftz antibody¹¹ and an alkaline phosphatase-coupled second antibody preparation (Bio-rad).

peptides in yeast; each of these different GAL4/ftz chimaeric polypeptides could be detected in extracts of yeast cells with a polyclonal anti-ftz antibody¹¹ (Fig. 1*b*). The transcriptional activity seen with the GAL4/ftz chimaeras with either N- or C-terminal deletions of ftz coding sequences indicated that an activation domain of the ftz polypeptide might reside between amino acids 223 and 272. A GAL4/ftz fusion containing only this small domain (GAL4/ftz²²³⁻²⁷²) did not activate transcription however. Perhaps the transcriptional activation domain of ftz is not just a short array of contiguous amino acids as it is in GAL4 (ref. 14) and GCN4 (ref. 16).

If the ftz polypeptide is a transcriptional activator in *Drosophila*, then mutations that affect ftz function in *Drosophila* might be expected to alter the transcriptional activity of ftz in yeast. The dominant allele *Rpl* in *Drosophila* encodes a ftz polypeptide truncated at its C-terminus⁴. *Rpl* causes patchy transformations of the posterior third thoracic segment into the posterior second thoracic segment¹⁷. In yeast cells the GAL4/ftz^{Rpl} chimaeric polypeptide had about 25% of the transcription-activating potential of the wild-type GAL4/ftz

chimaera (Fig. 1*a*). This observation is consistent with the suggestion¹⁷ that a weakened ftz^{Rpl} polypeptide may be sufficient to define parasegmental boundaries but not sufficient to activate genes of the bithorax complex properly. We also assessed the effects in yeast of a second ftz mutation. The temperature-sensitive ftz^{147ts} allele encodes a single alanine to valine change within the homoeodomain of the ftz polypeptide⁴. In yeast cells the chimaeric GAL4/ftz^{147ts} polypeptide was a weak activator of transcription at high temperatures (30 °C or 37 °C; data not shown) and, somewhat unexpectedly, also at low temperatures (19 °C; Fig. 1*a*). As the ftz^{147ts} polypeptide does function at permissive temperatures in *Drosophila* embryos, it may be stabilized by interaction with other *Drosophila* transcription factors that are not present in yeast cells.

To determine whether the ftz polypeptide could also provide sequence-specific DNA-binding activity in yeast cells, we introduced into yeast cells a β-galactosidase reporter construct that contained six tandem repeats of a *Drosophila* ftz-binding chromosomal sequence⁸ as a transcriptional element (Fig. 2*a*). The results of β-galactosidase assays using this reporter con-

struct in yeast cells expressing GAL4/ftz³⁻⁴¹³, or a second GAL4ftz chimera (G/ftz³⁻⁴¹³) containing only the N-terminal 10 amino acids of GAL4, or GAL4 are shown in Fig. 2b. This oligomer of consensus ftz-binding sites functioned as a ftz-dependent UAS, with GAL4/ftz³⁻⁴¹³ and with the G/ftz³⁻⁴¹³ construct. Furthermore, the integrity of the homoeobox was necessary for this transcriptional activity. The deletion construct GAL4/ftz^{Δ274-302} which lacks 29 amino acids in the homoeobox, including its putative helix-turn-helix motif, did not activate transcription of this reporter gene. Nevertheless, this construct did provide some transcriptional activation function when UAS_G-dependent activity was monitored (Fig. 1a).

We have exploited the species interchangeability of transcription factors¹⁸⁻²² to establish that a *Drosophila* homoeodomain-containing regulator of embryonic development can act as a positive activator of transcription. That the transcriptional activation domains of GAL4 can be replaced by ftz sequences implies that GAL4 and ftz may stimulate transcription by a similar mechanism, perhaps by contacting RNA polymerase II (ref. 23) or the TATA-box factor²⁴ directly. Our deletion analyses (Fig. 1a) however, did not define any short acidic activation regions in the ftz polypeptide such as those in GAL4¹⁴. Transcriptional activation by the ftz polypeptide may therefore depend more on the overall secondary and tertiary structure of ftz, or on its post-translational phosphorylation¹¹. In preliminary experiments (V.D.F., H. M. Krause and C.J.I., unpublished observations) we have shown that the ftz polypeptide is also highly phosphorylated when it is expressed in yeast cells. These observations raise the possibility²⁵ that phosphorylation of serine and threonine residues may create acidic transcriptional activation domains in transcription factors such as ftz. The differential phosphorylation of ftz during *Drosophila* embryogenesis could be a fine-tuning mechanism for its potential as a transcriptional activator.

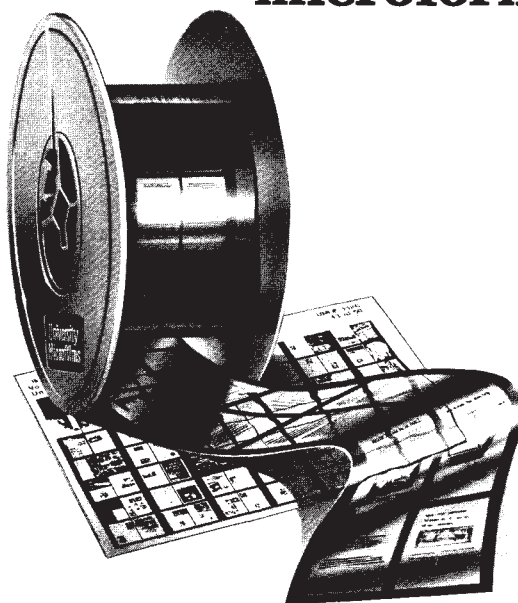
We thank Henry Krause and Jacqueline Segall for helpful suggestions, Henry Krause and Walter Gehring, Jun Ma and Mark Ptashne, Alan Laughon and Robert West for DNA, and Claude Desplans for the consensus ftz-binding element. This work was supported by grants from the MRC of Canada, the National Cancer Institute of Canada, and a grant from the NIH to the BIONET Resource. V.D.F. holds an MRC of Canada Studentship.

Note added in proof: two recent reports have demonstrated transcriptional activation by *Drosophila* homoeobox proteins^{30,31}.

Received 17 August 1988; accepted 9 January 1989.

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Computer-analysed 2-D electrophoresis

M.R. Krauss, P.J. Collins and S.H. Blose

A service is now available for analysing 2-dimensional gels and comparing them to existing databases, broadening the number of laboratories which can study protein expression using the technique.

Two-dimensional gel electrophoresis is a powerful biochemical resolving technique that allows life science researchers to investigate changes in cellular protein expression quantitatively. Using the appropriate protein identification procedures and experimental designs, the technique can be exploited to capture information on changes in genetic expression which can be compiled into databases of protein information. Powerful pattern-matching and data analysis software has been developed to harness and manipulate the large quantity of data contained in 2-dimensional gels, and new experimental approaches are being discovered to make the technique easier to perform.

Proteins identified by their abundance and positional coordinates serve as landmarks for the computer analysis of 2-dimensional gels. Valuable biological information can be gleaned by exposing cells to a set of specific experimental conditions and examining their biochemical response, provided the multitude of cellular reactions stimulated by the conditions can be deciphered. Protein databases compiling the global changes in cellular systems brought about during development, and by exposure to drugs, growth factors, and stress, can shed light on specific cellular biochemical mechanisms. The construction of such databases is becoming widespread by laboratories with scientists trained to carry out 2-dimensional gel electrophoresis, but for many labs the technology remains inaccessible.

Pathways to access

At Protein Databases, Inc. we have set up databases of protein information obtained by quantitatively scanning 2-dimensional gels. The NIH 3T3 cell database contains 28 named proteins separated using a 2-dimensional gel system with an isoelectric point range of pI 3.5-10; our human serum protein database has 257 identified proteins separated over the same range. The *Escherichia coli* database contains 98 named proteins separated using an equilibrium gel type, and 54 identified proteins separated on non-equilibrated gels.

We offer a range of levels of services for investigators interested in protein expression. At the most basic level, researchers may submit biological samples to Protein Databases, Inc. for analysis, using off-the-shelf sample preparation kits we have developed for cultured cells, solubilized tissue, and protein solutions.

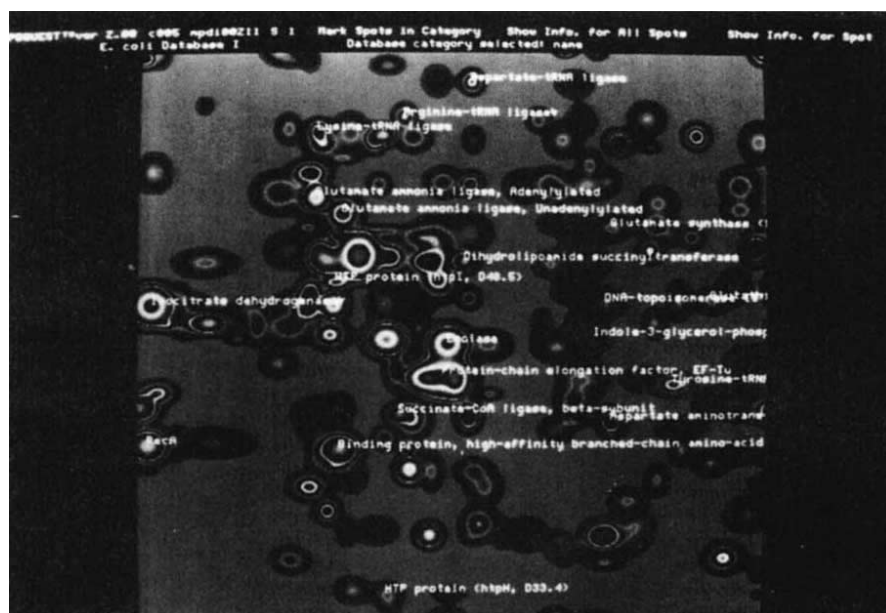


Fig. 1 Computer graphics screen showing part of the *Escherichia coli* database from Protein Databases, Inc., with several named proteins.

We perform high-resolution 2-dimensional electrophoresis, detect the proteins resolved in the gels using radioactive labels (3H , ^{14}C , ^{32}P , ^{33}P , ^{35}S , or ^{125}I isotopes), silver staining, or Coomassie blue staining, and submit the gels to computer-assisted analysis by The Discovery Series™ of densitometry hardware and pattern-matching software. The software compares the experimental gels to the database for that organism and identifies co-migrating proteins with known proteins in the database.

The PDQUEST™ software in The Discovery Series™ sorts the data to locate proteins which have increased, decreased, appeared or disappeared as a result of experimental manipulation. For each sample submitted, researchers receive multiple exposed films of the gels; a 3 ft × 3 ft wall map of all protein spots matched during analysis; tables quantifying the amount of protein in each spot; histograms comparing the amount of protein in each gel; scatter plots correlating the level of protein expression between gels; and a map of the position, relative molecular masses and isoelectric points of the 300 most abundant proteins.

Post-analysis services are available for sorting the data to answer specific questions, or researchers may request copies of the spreadsheet data on disks formatted for use with either Macintosh or MS-

DOS-based computers and perform their own analyses. We also offer pre-packaged services for performing phosphorylation studies, determination of partial amino acid composition, and *t* test and Mann-Whitney statistical analyses.

Researchers who perform 2-dimensional gel electrophoresis in their own laboratories can access our established databases by purchasing a sub-licence for the PDQUEST™ software and the pertinent protein database. Known proteins can be identified by comparing the 2-dimensional gels scanned and analysed with The Discovery Series™ system to proteins contained in the database. The PDQUEST™ software is available for MicroVAX, Sun and Masscomp computer systems running the UNIX operating system.

Researchers may also construct their own databases of protein information by purchasing The Discovery Series™ of hardware and software. After performing 2-dimensional electrophoresis and subjecting their gels to computer-assisted analysis, users can identify proteins of interest through a variety of biochemical means¹⁻³. The most common tools used today include blotting followed by various forms of specific identification, blotting followed by direct microsequencing of proteins from 2-dimensional gels⁴, 2-dimensional electrophoresis followed by deter-

mination of phospho- and glycoproteins⁵ and sub-cellular fractionation followed by 2-dimensional electrophoresis.

Protein Databases, Inc. also offers services for assisting researchers to purify and identify proteins of interest using preparative gels and blotting techniques that can provide purified protein in amounts sufficient for antibody identification or amino acid sequencing. The proteins are blotted onto either nitrocellulose or Millipore's Immobilon membranes. Blots to be used for direct microsequencing are first evaluated before the sequencing blots are prepared, and we consult with researchers to determine the feasibility of obtaining enough protein to sequence.

Genomic activity

Cellular protein databases permit the monitoring of gene activity by detecting the expression and regulation of individual proteins, and can be used to analyse the equivalent of two million base pairs of actively coding DNA. The construction of a database of protein information produces an inventory of parts which can be used to understand how the proteins work and interact. Complementary technologies such as protein sequencing and synthesis, *in vitro* and *in vivo* genetics, and gene sequencing and synthesis may be used to extract additional information.

The collected information can then be organized into broad categories: physiologic information (regulatory behaviour and function), biochemical information (metabolic and structural data), genetic information (gene mapping and the assignment of structural genes to the 2-dimensional gel map), and architectural information (location of the protein in the cell). A database with this information permits the location of genes coding for proteins of interest, and can aid in gene cloning, and the analysis of gene networks.

As science proceeds with the sequencing of various genomes — including the human genome — the elucidation of the structure and function of proteins expressed by specific genes will allow the scientific community to understand the subtle interplay of cellular systems which lead to the regulation of organisms as a whole. The major benefit of this will be to allow the modelling of the biochemical behaviour of the cell, which should permit predictions of system behaviour and new insights into various genomes. □

Marc R. Krauss, Paul J. Collins and Stephen H. Blose are at Protein Databases, Inc., 405 Oakwood Road, Huntington Station, New York 11746, USA. For more information, fill in reader service number 100.

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Software solutions

Programs for analysing chaos, keeping track of species density, word-processing mathematical expressions and teaching protein purification techniques are among this week's offerings.

DYNAMICAL Systems, Inc. has a set of software packages for studying **nonlinear systems and chaos** as mathematical models or as trends in experimental data gathered in any of the physical or social sciences (*Reader Service No. 101*). Dynamical Software I — now in its fourth release version — allows users to solve differential equations using an Adams-type integrator, iterate discrete maps, create 2- and 3-dimensional colour-coded phase portraits, and compute next amplitude, 1-dimensional, time-one and circle maps. Special features include data smoothing and interpolation, random number generation, and time series embedding using the Takens method. Version two of the Dynamical Software II package performs more sophisticated analyses: the integration of delay differential equations, the generation of power spectra for Rössler Attractors, and the computation of fractal dimensions, eigenvalues and eigenvectors, and Lyapunov exponents. DS I.4 costs \$250 (US), and DS II.2 costs \$350 (US); both can be purchased together for \$550 (US). The software runs on an IBM PC/AT/XT with a 1.2 Mbyte drive and a graphics card.

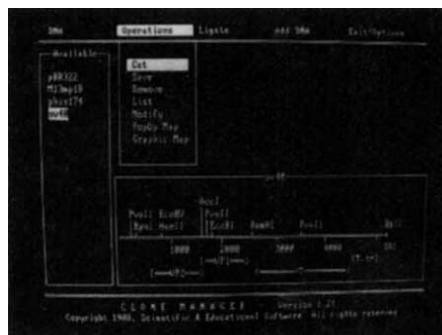
Wolfram Research is now distributing a **free program** for reading "Notebooks" generated using Stephen Wolfram's widely heralded *Mathematica* software, which contains a core of mathematical knowledge that can be used as an interactive calculational tool and as a programming language (*Reader Service No. 102*). The MathReader program is used for viewing interactive "Notebook" documents, which contain text, graphics and executable *Mathematica* commands. The "Notebooks" can serve as a medium for the exchange of technical documents or scientific results, and can be animated to form "movies" for tutorial purposes. MathReader allows users to view "Notebooks", to outline text and animate graphics, but not to edit or print documents. *Mathematica* also is now available for all 386-based MS-DOS computers, making it usable on virtually every computer system. (For a user review of *Mathematica*, see *Nature* 336, 319-20; 1988.)

Analytical applications

Tetrad, from Oxford Mobius, stores, processes, analyses and maps **species distribution** data for naturalist studies, using a 2 × 2 km square, or tetrad, as its unit of area (*Reader Service No. 103*). The program

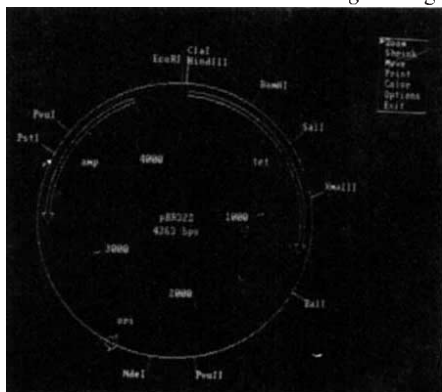
accepts up to 200 individual or species names for each database. Species information is stored on up to four levels in each tetrad, which can be analysed in any combination. Distribution maps can be produced directly from data files using a dot-matrix printer, eliminating the need for manual drawing or typing of data. The £74.75 (UK) Tetrad program runs on IBM PCs equipped with a hard disk with at least 1 Mbyte memory capacity.

The Clone Manager program from Scientific & Educational Software can be



The windows can be used to view linear representations of the map.

used to construct circular and linear **restriction maps** of recombinant DNA molecules (*Reader Service No. 104*). Fragments may be cut from any molecule and combined; the program automatically recalculates the base positions of restriction sites, genes and markers, and draws the new map. A special "PopUp" command opens a window on the screen to show the linear map of the DNA molecule being displayed. Cut DNA fragments are spliced together using the "Ligate" function: Clone Manager automatically recognizes blunt end ligations, and will relabel a fused *Bam*HI and a *Bgl*III frag-



Circular plasmid map generated using Clone Manager.

ment to acknowledge the creation of a new *Sau3A* site. The program is IBM-compatible and costs \$100 (US) for individual users.

BioMetallics sells its Δ -Soft software for the collection, analysis, storage, and reporting of data from endpoint and kinetic **microtitre assays** (*Reader Service No. 105*). The program offers five curve-fitting



The Δ -Soft kinetic ELISA software from BioMetallics displays the plate pattern together with the data.

options and a complete set of statistical operations. Δ -Soft can display the plate pattern simultaneously with the corresponding data, and it has a zoom function to allow users to inspect the kinetic data on individual wells more closely. The software will perform threshold screening and masking of specific wells, and will remove outliers automatically. The Macintosh-compatible program was designed for use with Molecular Devices microtitre plate readers, but can also be used with those from several other manufacturers.

The ChemResearch software from Isco, Inc. combines **HPLC gradient programming** and system control with dual-



The Isco ChemResearch software for data management and control, along with Isco HPLC hardware.

channel data acquisition and integration (*Reader Service No. 106*). The program runs on IBM PS/2 and PC-compatible computers, and the current version supports high-resolution colour graphics and the transfer of files to other applications. Data functions include real-time display and printout, integration reports and standards comparisons. ChemResearch was written for use with Isco HPLC solvent delivery systems, but it can also be adapted for use with binary gradients and with nearly any HPLC pump which has voltage or frequency inputs.

Hitachi's Spectra Calc software interfaces Hitachi scanning UV/visible, near-infrared and fluorescence spectrophotometers with IBM PCs and compatibles (*Reader Service No. 107*). The software provides an array of commands for **spectral data processing**, including a "Draw" function which allows the use of lines, arrows, boxes, icons, and different fonts and colours for the presentation of data. Hitachi says Spectra Calc's Array Basic programming language runs at speeds associated with mini-computers, and allows users to write customized software. Files created with Spectra Calc can be converted to a variety of formats, including ASCII, Lotus, DIF and J-Camp.

Axiom Chromatography is offering a free IBM-compatible demonstration disk for its range of PC-based **chromatography data systems** to give researchers interested in the software a chance to test it out in their own laboratory (*Reader Service No. 108*). The company offers systems which can be configured for the integration of HPLC, GC, supercritical fluid chromatography, isocratic chromatography and capillary zone electrophoresis.

Scientific publishing

Vuman Computer Systems's VuWriter **word-processing software** provides full Greek, scientific and teletex character sets, and allows the construction of brackets and integral signs of any specified height (*Reader Service No. 109*). The £95 (UK) program has an on-screen overstrike facility through which complex characters can be built, and the Greek character set has diacritics for printing Western European language characters. VuWriter can now be linked to the Aldus PageMaker and the Xerox Ventura desktop publishing systems.

EndNote, released by Niles & Associates last year, automates **bibliography building** and the formatting of in-text citations (*Reader Service No. 110*). The software is a combination of a desk accessory and an applications program, and is designed to work with Microsoft Word, MacWrite and WriteNow word-processing software. EndNote can maintain searchable databases of up to 32,000 references, and users can paste citations into text as they write, without leaving the computer to search through journals. EndNote scans the finished paper and creates a bibliography of cited works at the end of the paper, in the correct styles for *Nature*, *Science* or several other leading journals. The non-copy-protected software sells for \$129 (US).

Two new updates are available for the Reference Manager **bibliography management software** from Research Information Systems (*Reader Service No. 111*).

Reference Manager now has a Splicer module that can splice citations located using the software directly into word processing text, through a series of cut-and-paste operations. The Splicer module requires Reference Manager 4.0 or higher, and 210 Kbytes of available RAM after loading the word-processing program. Through the Reference Update service offered by the company, users receive a weekly diskette containing current information from over 400 of the leading biological and medical journals. The updates can be searched for combinations of authors, journal names and phrases, and identified references can be printed out as lists or reprint request cards, or can be exported to Reference Manager. Reference Manager is available for IBM-compatible and Macintosh PCs.

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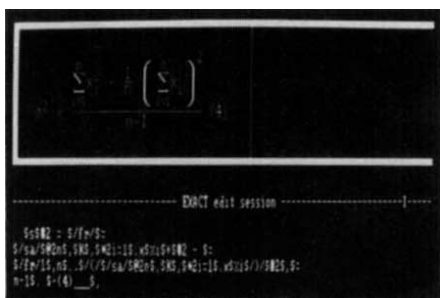


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The EXACT software from Technical Support Software, Inc. is for **mathematical typesetting** using Microsoft Word, WordStar, Multimate, Displaywrite, Samna, WordPerfect versions up to 5.0, and most other PC-based word-processors (*Reader Service No. 112*). EXACT is a RAM-resident program which cooperates with the user's word-processor to create complex mathematical expressions which can be displayed on the screen as they will appear in print. Through a split-screen function, EXACT can be used to edit mathematical expressions during the drafting of documents. Radicals, fractions, brackets and tables automatically rescale when they are changed: once the equation



With the EXACT mathematical software the command string and the equation can be viewed at once.

looks correct, the user can return to the text and insert the command string for printing the equation. The \$495 (US) basic IBM-compatible software (\$385 (US) for academics) includes 20 fonts with over 1,000 symbols and characters, plus a font editor for creating custom symbols.

Graphing and modelling

The French company Biostructure recently launched a computer-aided engineering package for evaluating the tridimensional structure of new molecules, and visualizing and manipulating them on a high-resolution graphics screen (*Reader Service No. 113*). Biostructure's BIO-EXPLORE software combines **molecular structure prediction tools** with interactive capabilities for searching the major international protein data banks to offer insights into new drug design. Protein engineers, immunologists and molecular biologists can use BIO-EXPLORE to identify homologies in known molecules and determine active sites of new molecules without performing experiments. BIO-EXPLORE is available as a turnkey system based on a Silicon Graphics workstation, starting at \$81,000 (US). The software is also available as an option on Silicon Graphics and DEC workstations.

Chemical Design offers an automated procedure for the construction of **3-dimensional models of proteins**, based on software techniques developed at Birkbeck College in London (*Reader Service No. 114*). The package predicts the 3-

dimensional structure of a protein by detecting homologies in its amino acid sequence with those corresponding to known protein folding patterns. Chemical Design says the approach has been used to generate useful models of proteins which shared only 20 per cent sequence homology with the protein that suggested its structure. The software matches the protein sequences and generates a number of 3-dimensional models automatically; the predicted structures can subsequently be manipulated and evaluated using molecular mechanics and dynamics programs. According to the company, the process of alignment and initial model building typically takes less than 10 minutes on a VAX 8600 computer.

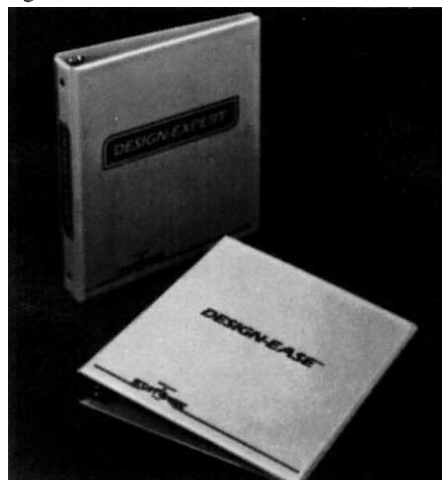
New England Software has published an **X-Y graphing utility** for technical and scientific applications which it says has the same user-friendly qualities as its award-winning Graph-in-the-Box software for making business graphs (*Reader Service No. 115*). The new ANALYTIC RAM-resident software makes 16 types of graphs based on X-Y and double-Y plotting, with a choice of linear or logarithmic scaling for up to three axes. Like its Graph-in-the-Box precursor, ANALYTIC captures data directly from the screen of any application program and instantly displays the data as a graph. The program offers high-low-close graphs, error bars, curve fitting and trend analysis; the graphs can be read against both a left and right Y-axis. The \$199.95 (US) IBM-compatible ANALYTIC can be used with most desktop publishing programs, and over 200 printers and plotters.

MicroMath's MINSQ package for **non-linear curve fitting and model development** can be used without data for plotting model equations, which can be optimized later to best fit data using least squares analysis (*Reader Service No. 116*). The models can be simple equations or multiple routines that can include parametric, integral or implicit functions. Model equations can be read from a file or entered from the keyboard, then modified interactively with the built-in editor. Up to 10 data sets may be present in memory simultaneously; previous models may be recalled for further analysis or plotting. Linear parameters are identified algebraically and solved for explicitly during the model fitting process, so that by default only nonlinear parameters are defined iteratively. Subsets of data or parameters may be fitted separately before fitting the whole model, and a "fine-tuning" option allows the adjustment of step sizes and convergence criteria for difficult problems. The interactive graphics section provides facilities for zooming in on smaller regions and for annotating plots with text, lines or arrows. The non-copy-

protected MINSQ requires MS-DOS 2.0 or greater, 512 Kbytes of computer memory and a graphics adaptor card, and costs \$179 (US).

Statistics packages

Stat-Ease, Inc. is now offering site licences for its **experimental design software** packages, DESIGN-EASE and DESIGN-



The DESIGN-EASE and DESIGN-EXPERT software packages from Stat-Ease offer statistical analysis capabilities for basic to specialized applications.

EXPERT, so that organizations can make copies of the programs for each individual's computer (*Reader Service No. 117*). DESIGN-EASE uses visual graphical techniques, and allows researchers to choose, conduct and analyse two-level experimental designs, including fractional factorial and Plackett-Burman designs. For the advanced experimenter, DESIGN-EXPERT offers response surface and mixture design capabilities. Stat-Ease says the program handles up to five responses simultaneously in its optimization routines, and Box-Behnken and central composite designs can be constructed and analysed. Both programs run on IBM computers and compatibles.

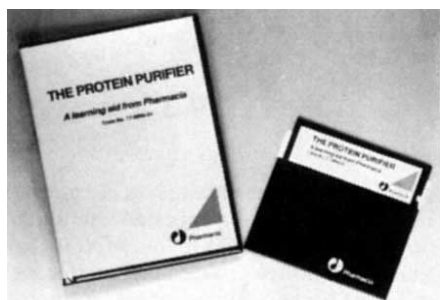
The SPSS Inc. **statistical data analysis** and presentation software is now available for the VAXstation 3100 and VAXstation 3520/3540 computers (*Reader Service No. 118*). SPSS-X offers a set of advanced statistical procedures which are used in business, government, and academic and scientific research. SPSS-Tables displays presentation-quality results of analyses or summarized data in several tabular forms. SPSS-Trends is a forecasting and time series analysis option including Box-Jenkins and spectral analysis modeling techniques. SPSS intends to develop SPSS-X software to support the DECstation 3100 this year. The company has also signed an agreement with Management Computer International AB of Stockholm, Sweden to jointly develop a statistical data analysis package for the Apple Macintosh II and SE computers by mid-1989.

SYSTAT has just released its **FASTAT statistics and graphics package**, for the Macintosh computer (*Reader Service No. 119*). FASTAT's list of statistical feats includes one-way and multi-way tables, Chi-square, Pearson and Spearman correlations, regression, non-parametric tests, series transformation and smoothing, forecasting, principal components and common factor analysis, and travelling salesman problems. The \$195 (US) software will create a sheaf of sophisticated graphs — many through only a click of the mouse — including stem-and-leaf diagrams, bubble plots, scatterplot matrices, factor-loading plots, and 2- and 3-dimensional scatterplots. MacDraw-like tools can be used to adapt and colour graphs, and scatterplot brushing tools are available for isolating and extracting cases, and "zooming in" on point clouds.

The PARVUS software package from Elsevier Scientific Software is a set of 50 programs for **multivariate statistical data analysis** (*Reader Service No. 120*). The IBM-compatible package is intended for general pattern recognition, and includes groups of programs for the explorative analysis and representation, classification and correlation of data. The programs can be divided into several functional groups: data import, data manipulation and pre-processing, methods classification, correlation analysis, target factor analysis, regression analysis, nonlinear mapping, graphical presentation, and utilities. The modular structure of PARVUS allows it to be used in a variety of ways, depending upon the application — from palaeontology to oenology. PARVUS costs \$645, and comes on 15 diskettes supported by an extensive program manual.

Tutorials

The Protein Purifier from Pharmacia LKB is an IBM-based interactive program for teaching basic **protein purification techniques** (*Reader Service No. 121*). Students choose to purify one out of a starting



Pharmacia LKB's Protein Purifier tutorial program leads students through the purification process.

mixture of 20 proteins, using precipitation, gel filtration, hydrophobic interaction, chromatofocusing and other techniques. The software generates a chromatogram, showing the results of the separation, and

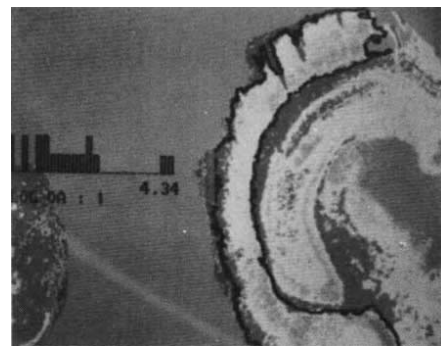
efficiency tables to record how many man-hours per 100 units of international activity were required. Protein Purifier simulates activity tests to show active fractions, the pooling of tubes, and electrophoresis for determining the level or purity or certain physical characteristics.

Biosoft's Neurosim is a package of four programs which **simulate neural function** on an IBM PC (*Reader Service No. 122*). The user selects experimental and neurophysiological parameters, and the screen output resembles that of an oscilloscope during genuine experiments. Combinations of parameters can be saved as disk files, so instructors can set up experiments for subsequent demonstration. Any screen can be printed out on an Epson-compatible printer. Neurosim is comprised of four modules: Cable simulates passive conduction in the long length of an axon; HH simulates the Hodgkin-Huxley equations for the production of an action potential, and can be run clamped or unclamped; PSP shows the post-synaptic potentials developed by a neuron in response to various types of synaptic input; and Network simulates the rhythmic properties of a simple neural network of five neurons. The \$199 (US) Neurosim package is for IBM PCs and true compatibles equipped with a graphics card.

TransTech has an educational/research package of software and plastic models for the investigation of **nuclear structure theory** using IBM-compatible computers (*Reader Service No. 123*). The software that comes with TransTech's face-centered cubic, or FCC, model package is designed to make quantitative comparisons among various nuclear structure models, and to elucidate the geometry of the solid-phase FCC model. The graphical displays emphasize the solid-phase FCC model and demonstrate how the liquid-drop, shell and cluster aspects of nuclei can simultaneously be found within the FCC model. TransTech says the package should also be useful for physicists involved in quark studies, because the FCC model is fundamentally a variant of the independent-particle model. TransTech sells the FCC package for \$180 (US) or £90 (UK).

Image analysis

Sight Systems has developed a special software package for the automatic **analysis of autoradiographs** — the Image Manager (*Reader Service No. 124*). Image Manager offers an alternative to calibrating autoradiographs by comparing grey images with the reference exposures at the bottom of the film. With Image Manager, a video camera is used to capture an image of the autoradiograph, which is then digitized and stored on the system's 1,500-image capacity hard disk. When recalled



An autoradiograph of a rat brain processed using Image Manager, showing opiate receptors.

for analysis, the dosage levels of selected areas of the autoradiograph can be accurately determined. Greyscales reflected on the autoradiograph can be allocated pseudo colours on the screen for enhanced readability. Results can be graphed in several different formats, and can be transferred as ASCII files to other software packages for further analysis.

JAVA, from Jandel Scientific, is an IBM-compatible software package designed to **measure and analyse video images** from composite video sources, such as video cameras and VCRs, and pre-digitized formats, such as CAT, MRI and ultrasound (*Reader Service No. 125*). In addition to image processing, JAVA's capabilities include densitometry, morphometric analysis, automatic line and edge



The JAVA software from Jandel measures and analyses images from a variety of sources.

digitizing, and automatic object counting. Measurements are targeted to a 64,000-row by 16,000-column virtual worksheet which includes descriptive statistical functions and data transforms. JAVA now supports both the 640-pixel and 512-pixel versions of the Imaging Technology, Inc. PCVISION^{plus} line of video digitizing boards, or frame grabbers. JAVA costs \$995 (US).

The HIPS **general-use image processing package** from SharpImage Software runs under the UNIX operating system, and is compatible with VAX, MicroVAX, Sun, Apollo, Masscomp, NCR Tower, Iris and IBM AT computers (*Reader Service No. 126*). HIPS handles sequences of images in the same manner in which it processes single frames. Programs have been devel-

oped for simple image transformations, filtering, convolution, Fourier and other transform processing and edge detection. Users can also integrate their own custom routines. SharpImage says the \$2,995 (US) HIPS can be adapted for use with most image display devices.

The supercomputer company Stellar has announced 30 new programs for its Stellar **graphics supercomputer**, including two new packages for image processing (*Reader Service No. 127*). Stellar says its supercomputer's dynamic 3-dimensional graphics capabilities and architecture provide powerful tools for real image proces-



The Stellar Graphics Supercomputer has 30 new software packages.

sing in the fields of medical imaging, seismic modelling, remote sensing and scientific visualization. The new image processing programs are the IM Raster Toolkit from the University of Waterloo for the manipulation of raster images, and the Astronomical Image Processing System from the US National Radio Astronomy Observatory. Programs for MCAE, ECAD, computational chemistry, geophysical studies, animation and computational fluid dynamics are also available.

Database data

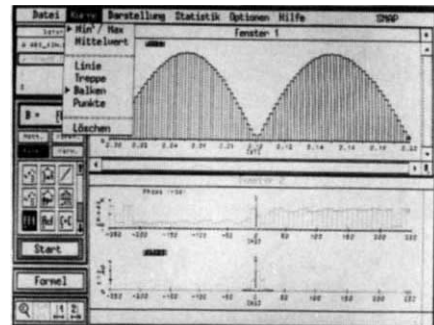
ChromHELP, Inc. offers an **HPLC/TLC database** which allows busy chromatographers to find the latest and best separation methods without searching through piles of journals (*Reader Service No. 128*). The ChromHELP database contains over 3,000 references for the separation of over 7,000 compounds, compiled from journal articles published from 1987 to 1989. All entries include title, author, references and keywords, as well as the column size and packing, the mobile phase and flow rate, and important parameters pertaining to detection of the compound. The ChromHELP database is free to purchasers of ChromHELP, Inc. columns; others pay a nominal charge for searches and printouts.

CAIRS Information Management Systems now offers a facility for immediate updating on its **CAIRS text retrieval**

database for micro-to mainframe computers (*Reader Service No. 129*). Until this development, new information entered on CAIRS, or modifications of existing records, were held in a holdings file and merged with the main database at periodic intervals, usually the end of the day. With the new facility, specific text in an existing record can be changed without having to go through the update process, and exposing data which must never be changed to an update procedure.

Data links

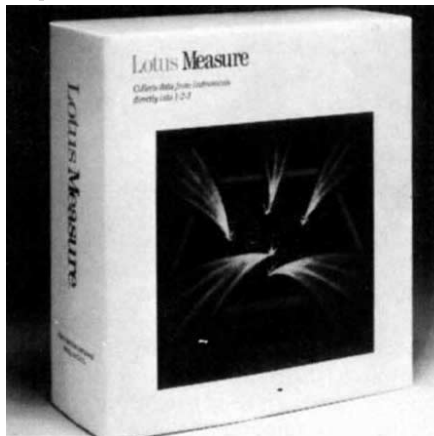
The Personal Computer Instruments-Signal Analysis Package, or PCI-SNAP, from Siemens is a **mathematical software package** for evaluating and analysing measured data from PC-based instruments (*Reader Service No. 130*). PCI-SNAP runs on any IBM-AT or XT-compatible PC with MS-DOS higher than version 2.1, and accepts data in ASCII files as well as other formats. The opera-



The German version of Siemens' PCI-SNAP for the mathematical analysis of data.

tion of PCI-SNAPS is graphics-oriented: the user can perform more than 100 functions and operations using the mouse, windowing, pull-down menus and graphic symbols. File management and the output of measured data is also controlled through these user interfaces.

The UK distributor of Lotus Measure, Industrial Data Processing Ltd, is now giving hands-on courses on how scientists and engineers can adapt the software using a



Lotus Measure can be adapted for use with scientific and engineering applications.

PC for their own **data acquisition and analysis** applications (*Reader Service No. 131*). Topics to be covered include interfacing with instruments, sensors, plug-in boards and A/D devices, real-time data analysis and display, fast Fourier transforms, graphical presentations such as trend and mimic displays, curve fitting and statistics, communicating data direct to a spreadsheet, and preparation of technical data for publication. Lotus Measure controls and monitors experiments and other processes by sending commands to instrumentation and capturing data directly from sensors and instruments connected through IEEE-48 bus and RS-232C serial interfaces.

Dedicated programs

The Gene-Master **DNA analysis program** for the Gene-Master DNA Workstation from Bio-Rad prompts the user for all parameters and sequence files required in the analysis (*Reader Service No. 132*). Version 2.0 includes the Lipman-Pearson FASTN and FASTP functions for fast alignments and similarity searches of DNA and protein databases. Bio-Rad says it also locates any word in GenBank or NBRF-PIR annotations in less than five seconds, double-checks entries for gels entered by sonic digitizer, and inserts whole sequence files into another specified sequence to simulate genetic engineering experiments. The program includes the Staden Shotgun Handler, which graphically displays sequence overlaps.

The TA72 GraphWare software from Mettler supports the processing of **thermo-analytical measured values and curves** determined by the Mettler TA3000 system (*Reader Service No. 133*). The software



Mettler's thermal analysis software.

allows several thermoanalytical curves to be compared and processed simultaneously. Other features include full or partial integration over a definable range with different baseline types, onset determination with tangents, and content analysis for crystallinity. □

These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.